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THYROID HORMONE HOMEOSTASIS IN THE PERINATAL MOUSE BRAIN: IMPLICATIONS FOR MCT8 TRANSPORT DEFECT

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Memoria que presenta la licenciada en Biología **Soledad Bárez López** para optar al grado de Doctor Internacional por la Universidad Autónoma de Madrid

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ABSTRACT / RESUMEN

ABSTRACT

Thyroid hormones (TH) play an essential role both in the developing and the adult central nervous system (CNS). The concentrations of the genomically active hormone T3 in the brain depend on TH transport across the brain barriers, the local generation of T3 in astrocytes by type 2 deiodinase (D2), and TH degradation by type 3 deiodinase (D3). In addition, during foetal development, circulating TH concentrations derive partly from the mother. How all these factors interact during mice foetal development to ensure "brain euthyroidism" is not well known.

This work has explored the sources of T3 that ensure brain euthyroidism during mice foetal development by treating euthyroid pregnant dams with T4 or T3 from embryonic day 12 (E12) to E18. The outcome of the treatment revealed that a large amount of foetal T4 during late gestation is of maternal origin and that maternal-foetal T3 transfer is tightly regulated, most probably at the placental level. Also, that T4 transport across the choroid plexus along with D2 activity at the blood-cerebrospinal fluid barrier might be key events in TH action in brain during foetal development, as conversion of T4 into T3 by D2 activity might be the only source of T3 during mouse brain development.

Mutations in the gene expressing the TH monocarboxylate transporter 8 (MCT8), in humans, lead to altered circulating TH levels and a severe neurodevelopmental disorder. Due to the crucial role of this transporter, the interaction between Mct8-dependent transport and D2 activity was studied. D2 activity was found to be required to generate the brain hyperthyroidism characteristic of Mct8 deficient (*Mct8*KO) animals during perinatal stages of development.

Besides, *Mct8*KO mice were used to test novel therapeutic approaches as possible treatments for MCT8 deficiency. The use of the TH analogue triiodothyroacetic acid (TRIAC) restored plasma T3 levels but severely decreased T4 levels leading to a state of brain hypothyroidism with reduced T3 content. Delivery of MCT8 by adeno-associated virus vectors proved that, for a successful gene therapy, restoration of MCT8 at the brain barriers is critical to mediate TH access to the brain of MCT8 deficient patients. Finally, intranasal delivery of TH as an alternative administration route increased TH levels in the systemic circulation, aggravating the peripheral hyperthyroidism, suggesting that is not a treatment option for MCT8 deficient patients.

RESUMEN

Las hormonas tiroideas (HT) juegan un papel esencial en el desarrollo y función del sistema nervioso central. En el cerebro, las concentraciones de la hormona activa a nivel genómico, T3, dependen del transporte de HT a través de las barrera cerebrales, de la generación local de T3 en los astrocitos mediante la desyodasa tipo 2 (D2) y de la degradación de HT por la desyodasa tipo 3 (D3). Además, durante el desarrollo fetal las concentraciones de HT circulantes provienen en parte de la madre. Cómo interactúan todos estos factores durante el desarrollo fetal en ratón para asegurar un "eutiroidismo cerebral" no se conoce suficientemente.

En este trabajo se han explorado las fuentes de T3 que posibilitan un eutiroidismo cerebral durante el desarrollo fetal del ratón mediante el tratamiento de madres gestantes con T4 o T3 desde el día embrionario 12 (E12) hasta E18. El resultado del tratamiento reveló que una gran parte de la T4 fetal durante las fases finales de la gestación es de origen materno y que la transferencia materno-fetal de T3 está estrictamente regulada, probablemente a nivel placentario. También, que el transporte de T4 a través del plexo coroideo junto con la actividad de D2 en la barrera sangre-líquido cefalorraquídeo podrían ser eventos claves en la acción de las HT en el cerebro durante el desarrollo fetal, ya que la conversión de T4 a T3 mediante la actividad de D2 podría ser la única fuente de T3 durante el desarrollo cerebral del ratón.

Mutaciones en el gen que expresa el transportador de HT, transportador de monocarboxilatos 8 (MCT8), provocan alteraciones en los niveles circulantes de HT y discapacidades graves del neurodesarrollo en humanos. Debido al papel fundamental de este transportador, se estudió la interacción entre el transporte dependiente de Mct8 y la actividad de D2. Los resultados indican que la actividad de D2 es necesaria para generar el hipertiroidismo cerebral característico de los ratones deficientes de Mct8 durante etapas perinatales del desarrollo.

Además, se utilizaron ratones deficientes de Mct8 para examinar nuevas aproximaciones terapéuticas como posibles tratamientos para la deficiencia de MCT8. El uso del análogo de HT ácido triyodotiroacético (TRIAC) restableció los niveles plasmáticos de T3 pero redujo drásticamente los niveles de T4 generando un estado de hipotiroidismo cerebral con bajo contenido de T3. La administración de MCT8 mediante virus adeno-asociados demostró que, para una eficaz terapia génica, es crítico reestablecer MCT8 en las barreras cerebrales para mediar el acceso de HT en el cerebro de pacientes deficientes de MCT8. Finalmente, la administración de HT por vía intranasal como vía alternativa aumentó los niveles circulantes de HT agravando el hipertiroidismo periférico lo que sugiere que no es una opción para el tratamiento de pacientes deficientes de MCT8.

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ABBREVIATIONS

AAV9	Adeno-associated virus 9
AAVs	Adeno-associated virus
AHDS	Allan-Herndon-Dudley Syndrome
ANOVA	Analysis of variance
apoTR	Unligated thyroid hormone receptors
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BSA	Bovine serum albumin
BW	Body weight
chp	Choroid plexus
CNS	Central nervous system
CPu	Caudate Putamen/Striatum
CSF	Cerebrospinal fluid
D1	Type I iodothyronine deiodinase
D2	Type II iodothyronine deiodinase
D3	Type III iodothyronine deiodinase
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
DITPA	3,5-diiodothyropropionic acid
DTT	Dithiothreitol
E	Embryonic day
EV	Empty vector
FPKM	Fragments per kb of transcript sequence per million mapped fragments
FT4	Free T4
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hf	Hippocampal fissure
hMCT8	Human MCT8
HSA	Human serum albumin
I, II, III	Layers I, II, III of the cerebral cortex
ICV	Intracerebroventricularly
IQ	Intelligence quotient
IV	Intravenously
KO	Knockout
LAT1	Large neutral amino acids transporter small subunit 1
LAT2	Large neutral amino acids transporter small subunit 2
LO	Lateral olfactory tract
LT4	Levothyroxine
LV	Lateral ventricles
MCT	Monocarboxylate transporter
MCT10	Monocarboxylate transporter 10
MCT8	Monocarboxylate transporter 8
ME	Median eminence
mng	Meninges
NTCP	Sodium/Taurocholate Co-transporting Polypeptide
OATP	Organic anion-transporting polypeptide
P	Postnatal day

PB	Phosphate buffer
PBS	Phosphate-buffered saline
PHE	Phenylephrine hydrochloride
PTU	Propylthiouracil
PVN	Paraventricular nucleus
qPCR	Quantitative PCR
RIA	Radioimmunoassay
RT	Room temperature
rT3	3,3',5'-triiodothyronine
RTH	Resistance to thyroid hormone
RXR	9-cis retinoic acid receptor
SBP2	Sequence binding protein 2
ShMCT8	Short human MCT8
SLC16a2/Slc16a2	Solute carrier family 16 member 2 gene
T2	3,3'-diiodothyronine
T3	3,5,3'-triodo-L-thyronine
T4	Thyroxine or 3,5,3',5'-tetraiodo-L-thyronine
TBG	Thyroxine binding globulin
TH	Thyroid hormones
TRE	Thyroid hormone response elements
TRH	Thyrotropin-releasing hormone
TRIAC	3,3',5-triiodothyroacetic acid
TRs	Thyroid hormone receptors
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
Wt	Wild type

INTRODUCTION

1. Thyroid Hormones

Thyroid hormones (TH) are essential for the correct development of vertebrates controlling cell growth and metabolism in all tissues. The developing central nervous system (CNS) is particularly sensitive to TH deficiency as TH deficit leads to numerous alterations. The severity of these alterations depends on the timing of this deficit. While the wide range of clinical manifestations of adult onset hypothyroidism are usually reversed with an appropriate treatment, TH deficit during prenatal development can lead to irreversible neurological alterations unless treated with a timely hormonal replacement therapy (Morreale de Escobar et al., 2000) highlighting the importance of both appropriate levels of TH and timing of action.

1.1 Synthesis, secretion and tissue distribution

TH are iodinated amino acids synthesised in the thyroid gland. Of the total hormonal secretion of the thyroid gland about 93% is as the prohormone thyroxine or 3,5,3',5'-tetraiodo-L-thyronine (T4) and 7% as 3,5,3'-triodo-L-thyronine (T3), the active form at the genomic level (Braverman and Utiger, 2000). The synthesis and secretion of TH are controlled by the hypothalamus-pituitary-thyroid axis. In the paraventricular nucleus (PVN) of the hypothalamus the thyrotropin-releasing hormone (TRH) is synthesised and secreted, which in turn stimulates the pituitary gland inducing the secretion of thyroid-stimulating hormone (TSH). TSH binds to its G-protein-coupled receptor in the thyroid gland and stimulates the biosynthesis of TH and secretion into the plasma. Both TRH and TSH are negatively regulated by TH (Chiamolera and Wondisford, 2009).

Most of the TH in the blood circulate bound to the transport proteins thyroxine binding globulin (TBG), transthyretin (TTR) and albumin (human serum albumin, HSA). Taking into account their molar concentrations as well as the association constants for T4, TBG binds 75% of serum T4, while TTR and HSA only bind 20% and 5%, respectively. In the serum approximately 0.03% of the total T4 and 0.3% of the total T3 are present in free or unbound form (Refetoff, 2015). The bound T4 is in rapid equilibrium with free T4 (FT4) that is available for cellular uptake. TH binding proteins allow T4 distribution through circulation and increase its half-life in blood serum (Schussler, 2000).

1.2 Metabolism

TH metabolic pathways include deiodination, sulfation, glucuronidation and oxidative deamination. Among these, sequential deiodination is the most important. Iodothyronine deiodinases are selenoproteins that catalyse the removal of iodide from TH. At first, TH deiodinases research focused on their role in maintaining normal serum levels of the biologically active molecule T3. Years later it was demonstrated that these enzymes can locally modify TH tissular bioactivity independently of the concentrations in serum revealing that deiodinases play a much more important role than initially thought (Visser and Peeters, 2012). Three different types of deiodinases have been identified, with different catalytic specificities, tissue distribution, physiological functions, and regulation.

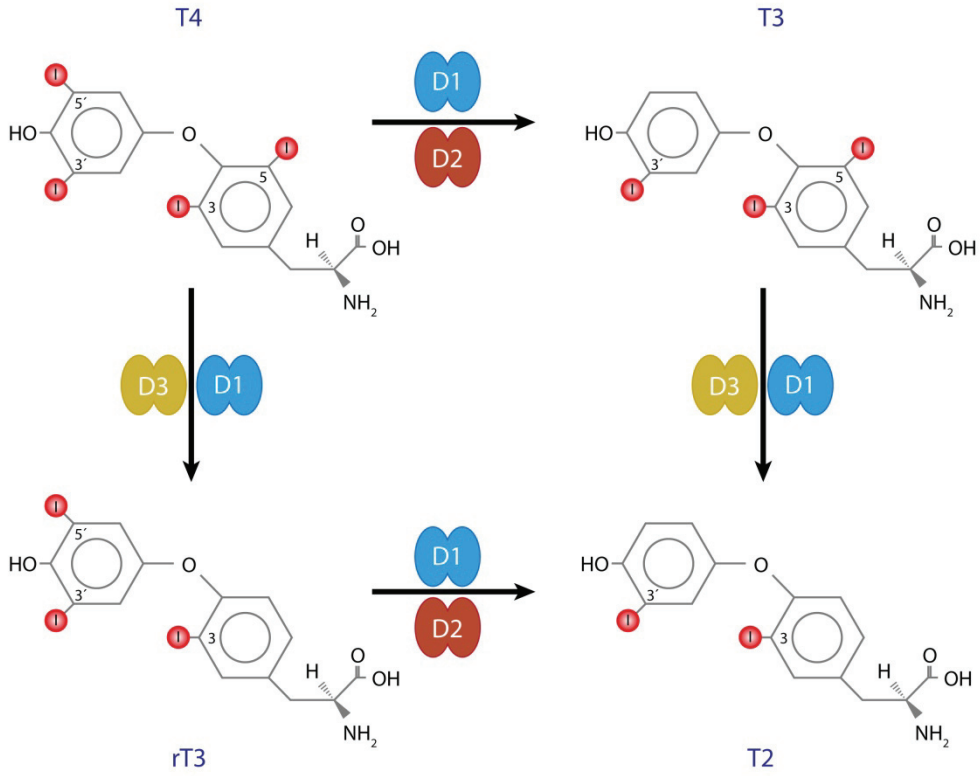


Figure 1. Thyroid Hormone structure and metabolism. Iodothyronines are molecules made of two phenolic rings bound by an oxygen atom and contain between 2 and 4 iodine (I) atoms. Sequential deiodination is the most important metabolic process mediated by deiodinases D1, D2 and D3 that catalyse the removal of iodide from TH.

Type I iodothyronine deiodinase (D1, encoded by *Dio1*) is the only deiodinase with both outer- (5') and inner (5)-ring activities and catalyses the conversion of T4 to the genomically active

T3 or to the genomically inactive metabolite reverse T3 (3,3',5'-triiodothyronine, or rT3). It can further catalyse T3 and rT3 to 3,3'-diiodothyronine (T2), also an inactive metabolite at the genomic level (Bianco and Kim, 2006) (**Figure 1**). D1 is expressed predominantly in tissues with rapid exchange with blood like liver, kidney and thyroid follicular cells and it is most likely localised at the plasma membrane of cells (Baqui et al., 2000). D1 activity is believed to be a major source of circulating T3. Nevertheless, the high affinity towards rT3 and other inactive metabolites suggests a role in the recovery and recycling of iodine within the organism (Schneider et al., 2006). Among the different factors that regulate D1 activity the most important one is the regulation by T3. T3 induces *Dio1* expression, and this aggravates the T3 excess commonly found in hyperthyroidism (Koenig, 2005).

Type II iodothyronine deiodinase (D2, encoded by *Dio2*) catalyses via outer (5')-ring deiodination the prohormone T4 into the active metabolite T3, and rT3 into T2 (**Figure 1**) (Bianco and Kim, 2006). D2 is localised at the endoplasmic reticulum of cells (Baqui et al., 2000) in tissues with slow exchange with blood and where local regulation of the intracellular concentration of T3 is critical such as the brain, anterior pituitary, brown adipose tissue, skeletal muscle, thyroid and heart (Lavado-Autric et al., 2013). D2 activity is regulated by T4 at the post-translational level by inducing protein degradation at the proteasome (Steinsapir et al., 1998). To a lesser extent, D2 can also be regulated at the transcriptional level with T3 inhibiting *Dio2* expression (Croteau et al., 1996) and with T3 deficit increasing *Dio2* expression (Guadaño-Ferraz et al., 1999).

Type III iodothyronine deiodinase (D3, encoded by *Dio3*) catalyses the inner (5)-ring deiodination of T4 and T3 into rT3 and T2 respectively, inactivating TH action (Bianco and Kim, 2006) (**Figure 1**). D3 is expressed in brain, skin and foetal tissues such as the uterus and the placenta suggesting an important role in protecting the foetus from an excess of T3 (Bates et al., 1999, Kester et al., 2004). At the cellular level it is present at the plasma membrane (Baqui et al., 2003). D3 is transcriptionally regulated by T3 inducing *Dio3* expression (Barca-Mayo et al., 2011), however, it is also regulated by other factors such as oestrogen and progesterone, growth factors, glucocorticoids and growth hormone or even genomic imprinting (Reviewed in Hernandez (2005)).

In the brain deiodinases play an essential role in maintaining appropriate levels of T3. D2 and D3 in the brain are developmentally regulated as shown during the rat early postnatal development where the maturational patterns of D2 and D3 activities reveal a progressive

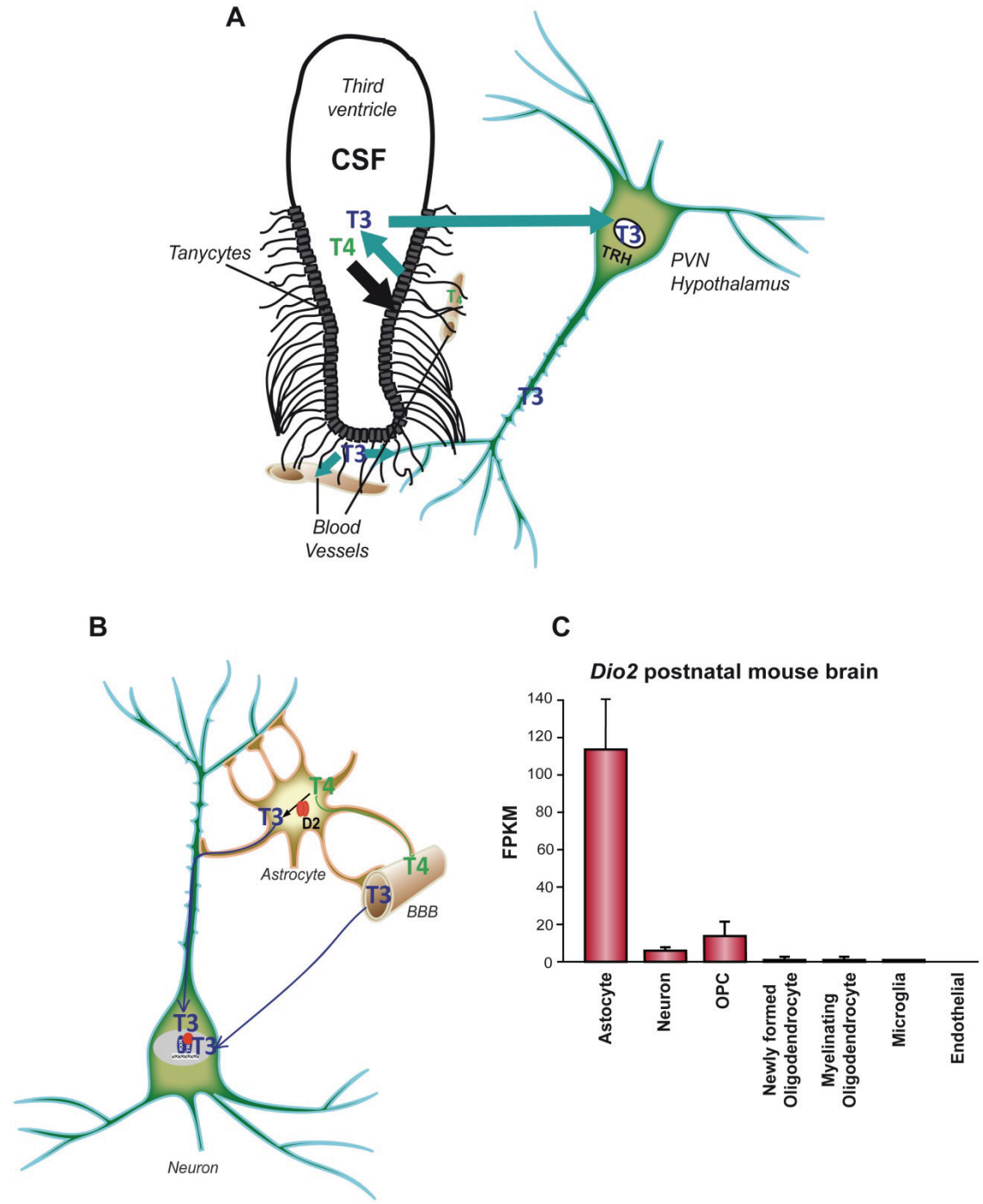


Figure 2. **Deiodinase type 2 (D2) in the brain. (A)** Schematic representation of T3 availability to the paraventricular nucleus (PVN) of the hypothalamus and to blood vessels in the median eminence. T4 from the cerebrospinal fluid (CSF) or the blood vessels enters the tanycytes lining the third ventricle where it gets converted into T3 by D2 activity. T3 is released into the CSF for conveyance to the PVN by diffusion or directly through the tanycytes processes. **(B)** Schematic representation of T3 availability to neurons through the blood-brain-barrier (BBB). T4 from the BBB enters the astrocytes where it gets converted into T3 by D2 and transported into the neurons. **(C)** *Dio2* mRNA expression in the postnatal mouse brain, from transcriptomic analysis of purified native cell populations using RNA-sequencing (Zhang, 2014). The data represent the relative abundance of RNA molecules expressed as RNA fragments per kb of transcript sequence per million mapped fragments (FPKM).

increase in D2 activity while D3 activity follows the opposite pattern (Kaplan and Yaskoski, 1981). In adults *Dio3* is expressed in neurons throughout the brain (Tu et al., 1999) while *Dio2* is expressed in the glial cells astrocytes and tanycytes (Guadaño-Ferraz et al., 1997, Tu et al., 1997). Tanycytes line the lower third of the walls of the third ventricle and send their processes to the PVN of the hypothalamus and to the median eminence where they frequently end in blood vessels. Therefore D2 in the tanycytes could provide T3 to the cerebrospinal fluid (CSF) from where it could diffuse to nearby structures and to the hypothalamic nuclei directly through the tanycytes processes and control TRH production (**Figure 2 A**). On the other hand, D2 in the astrocytes is believed to be involved in generating and delivering T3 to nearby neurons (Guadaño-Ferraz et al., 1997) (**Figure 2 B**). Recent transcriptome analysis of mouse cerebral cortex has confirmed the expression of *Dio2* mRNA in astrocytes; in some neurons verifying early indications for expression in some interneurons, especially in hypothyroid conditions (Guadaño-Ferraz et al., 1999), and has additionally detected *Dio2* at oligodendrocyte precursor cells (Zhang et al., 2014) (**Figure 2 C**).

1.3 Thyroid hormone transporters

Because of the lipophilic nature of TH, it was believed that they could cross the cell membrane through passive diffusion. Then, in 2004 the description of mutations in a gene encoding for a cell membrane TH transporter that led to severe neurological alterations, established the importance of TH transport (Dumitrescu et al., 2004, Friesema et al., 2004). To date, several families of transporters with the capacity to transport TH have been described: monocarboxylate transporters (MCTs), sodium/taurocholate co-transporting polypeptide (NTCP), L-type amino acid transporters and organic anion-transporting polypeptide (OATPs).

- **MCTs**

The MCTs are proteins of 426 to 565 amino acids with 12 transmembrane domains, which transport monocarboxylates like pyruvate, lactate, ketone bodies, carnitine and aromatic amino acids. Most of the MCTs take up the substrates together with a proton, and they can also facilitate efflux of their ligands, however, two members of this family function as proton-independent TH transporters: MCT8 and MCT10 (Halestrap, 2012).

MCT8 (encoded by *SLC16A2*) is highly specific for T3 and T4 transport, but it can also transport rT3 and T2 (Friesema et al., 2003). Due to the remarkable pathophysiological

manifestations that arise from mutations in MCT8, this transporter will be described in further detail below.

MCT10 (encoded by *SLC16A10*) transports aromatic amino acids as well as T3 and T4. It is slightly more efficient than MCT8 in transporting T3, and less efficient in transporting T4. In the mouse brain, *Slc16a10* is expressed in neurons and in neural cells in the white matter (Müller and Heuer, 2014).

- **NTCP**

NTCP (encoded by *SLC10A1)* or the liver sodium/taurocholate cotransporter, is a seven transmembrane domain glycoprotein that belongs to the family of sodium-dependent organic anion transporters. It is expressed in hepatocytes and its main function is the transport of conjugated bile acids in liver, but it can also transport unconjugated bile acids and other amphipathic compounds. Furthermore, transport of the iodothyronines T4, T3, rT3, and T2, as well as their sulphated forms T4S and T3S, has been described for this transporter (Friesema et al., 1999).

- **L-type amino acid transporters**

The L-type amino acid transporters are heterodimeric proteins that consist of a heavy and a light chain linked through a disulfide bond. The light chain possesses 12-transmembrane domains while the heavy chain presents a single transmembrane domain. These proteins mediate the transport of neutral amino acids like leucine, phenylalanine and tyrosine. The sodium-independent transporters LAT1 (large neutral amino acids transporter small subunit 1, encoded by *SLC7A5*) and LAT2 (large neutral amino acids transporter small subunit 2, encoded by *SLC7A8*) can transport as well T4 and T3 (Friesema et al., 2005). In the developing mouse brain *Lat1* and *Lat2* mRNA are largely expressed in neurons, oligodendrocyte precursor cells, microglia, and are very abundant in endothelial cells of the blood-brain-barrier (BBB). *Lat1* mRNA is also present in the astrocytes (Bernal et al., 2015).

- **OATPs**

The OATP transporter family comprises proteins with 12 transmembrane domains responsible for the sodium-independent transport of amphipathic organic compounds, such as steroids, bile salts, drugs and anionic oligopeptides. Studies *in vitro* have revealed that at least 7 human OATPs and 14 mouse OATPs can mediate TH transport. The mouse postnatal brain expresses the transporters Oatp1c1 (encoded by *Slco1c1*), Oatp1a4 (encoded by *Slco1a4*) in endothelial

cells, Oatp3a1 (encoded by *Slco3a1*) in oligodendrocytes and Oatp2b1 (encoded by *Slco2b1*) in endothelial cells and microglia. Of especial relevance is the Oatp1c1 transporter that transports T4, rT3 and T4S with high specificity, and in the developing mouse brain, is present in astrocytes and endothelial cells of the BBB and the choroid plexus (Bernal et al., 2015, Roberts et al., 2008, Sugiyama et al., 2003).

1.4 Mechanism of thyroid hormone action

The main pathway of TH action is at the genomic level regulating gene expression through the binding of T3 to its nuclear TH receptors (TRs) which function as ligand-activated transcription factors. TRs belong to the nuclear receptor superfamily and are encoded by two different genes, *THRA* and *THRB* in humans (*Thra* and *Thrb* in rodents). *THRA* encodes the isoforms TRα1, TRα2, ΔTRα1 and ΔTRα2 and *THRB* encodes TRβ1 and TRβ2. Only three TRs display full receptor function at the genomic level: TRα1, TRβ1 and TRβ2. They present three distinct domains: an amino-terminal domain that is involved in the regulation of transcription, a DNA binding domain that is a highly conserved domain that recognises the TH response elements (TRE) in the DNA and a carboxyl-terminal ligand-binding domain that binds TH as well as coregulators that can be either corepressors or coactivators (Cheng et al., 2010). The receptor can bind the DNA as a monomer, as homodimer or most frequently as heterodimer with the 9-cis retinoic acid receptor (RXR) (Force et al., 1994).

Genes that are induced by T3 and repressed in hypothyroidism are identified as “positively regulated genes” while genes repressed by T3 and induced in hypothyroidism are defined as “negatively regulated genes”. All of them will be referred as T3-dependent or T3-responsive genes.

When TRs are bound to the DNA in absence of T3 (unliganded TR or apoTR), T3-dependent genes display basal levels of transcription. For positive target genes, in the absence of T3, the heterodimer RXR-apoTR is bound to the TRE where it interacts with different corepressor proteins with histone deacetylase activity that compact the chromatin repressing transcription (Grunstein, 1997). Upon the binding of T3 to the TR, there is a conformational change in the receptor that leads to the release of corepressor proteins and the recruitment of coactivator proteins with acetylase activity that in turn activate the transcription of the target gene (Li et al., 1999). In the case of negative regulated genes, the basal levels of transcription are inhibited upon the binding of T3 (Wang et al., 2009).

Because the ultimate effect of TH action is the regulation of gene expression, analysis of the expression of T3-dependent genes offers an additional tool to monitor the thyroidal status of different tissues. This approach has been widely used in the present work.

In addition to genomic actions, both T3 and T4 can exert non-genomic actions through receptors in the plasma membrane, cytoplasm or mitochondria. These receptors can have similar structures to those that mediate transcriptional actions of T3, like truncated TRα isoforms, or they can share no homologies with TRs such as the plasma membrane receptor integrin αvβ3. They can mediate processes such as angiogenesis, cell proliferation, intracellular microfilament organization or cellular respiration in the mitochondria expanding the amount of cellular events controlled by TH (Davis et al., 2016).

2. Thyroid hormone action in brain

2.1 Brain barriers

The passage of substances from the blood to the brain is regulated by the BBB and the blood-cerebrospinal fluid barrier (BCSFB). These barriers tightly control the influx and efflux of molecules and ions at the blood-brain interface to meet neuronal needs and to protect the brain from toxins and pathogens. The endothelial cells that compose the brain capillaries of the BBB have continuous intercellular tight junctions and contain few transcytotic vesicles which limit the passage of molecules through the endothelial cells. Thus, substances must leave the circulation and enter the brain parenchyma through transcellular transport (Obermeier et al., 2013). The astrocytes end-feet englobe the outer surface of the endothelium and regulate some features of the BBB (Abbott et al., 2006).

The BCSFB is formed by the epithelial cells lining the ventricular side of the choroid plexus. Studies in the rat revealed that the contribution of the choroid plexus to the total brain TH content is around 20% (Chanoine et al., 1992) and, since the BBB surface area is much greater than that of the choroid plexus, the BBB has been considered the major pathway for TH entry into the brain. However, the BCSFB could have a more important role during early development as it has been shown that the choroid plexus may have a greater transport capacity during early stages of brain growth and development (Keep and Jones, 1990).

2.2 Sources of thyroid hormones in the foetal and adult brain

In the brain several regulatory mechanisms control the concentrations of T4 and T3 in a very efficient fashion. These include secretion by the thyroid gland, TH transport to the brain, expression of deiodinases and, in the foetus, transplacental passage of maternal TH.

Studies in adult hypothyroid rats revealed that constant infusion of T3 at relatively low doses was enough to normalise T3 levels in peripheral tissues such as liver, kidney, heart or lung while much higher doses were necessary for the cerebral cortex to reach normal values. On the other hand, when T4 was administered, T3 values were normalised in the cerebral cortex at lower doses than those necessary to restore T3 levels in other peripheral tissues. Furthermore, T3 content in the cerebral cortex remained within the physiological range regardless of the increase in the dose of the T4 administered (Escobar-Morreale et al., 1999). This indicates that T4 is the primary source of T3 in the brain and that local conversion of T4 into T3 is tightly regulated avoiding excess of T3.

A model of T3 availability to neural cells has been proposed (Guadaño-Ferraz et al., 1997) and validated (Galton et al., 2007, Trajkovic et al., 2007, Dumitrescu et al., 2006). The model states that brain T3 has a double origin: a fraction is available directly from the circulation, and another is produced locally from T4 in the astrocytes by D2. In order to enter the brain, circulating T4 and T3 need to cross the brain barriers through specific TH transporters. Based on the location of these transporters the current model supports that (i) T3 and T4 cross the BBB through Mct8 into the extracellular fluid where they can directly reach the neural cells in the proximity of the blood vessels and that (ii) T4, but not T3, crosses the BBB through Oatp1c1 directly into the astrocytes through their end-feet in contact with the blood vessels, and produce additional T3 by D2 activity that can be then transported to the neuron (Morte and Bernal, 2014) (**Figure 3 A**). In primates, as OATP1C1 expression at the BBB is very low (Roberts et al., 2008), T4 transport through the BBB is believed to be mostly dependent on MCT8.

Studies in rodents suggest that each of these two routes contributes at least 50% to the total pool of brain T3 in the adult brain. D2 deficient mice at postnatal day 15 (P15) present around a 50% decrease in the content of T3 in the cerebral cortex (Galton et al., 2007) and 40% decrease at 3 months of age (Bárez-López et al., 2014). Furthermore, Mct8 deficient mice also present a 50% reduction in the content of T3 in the cerebral cortex (Trajkovic et al., 2007, Dumitrescu et al., 2006). Together these data suggest that in the postnatal brain of mice around

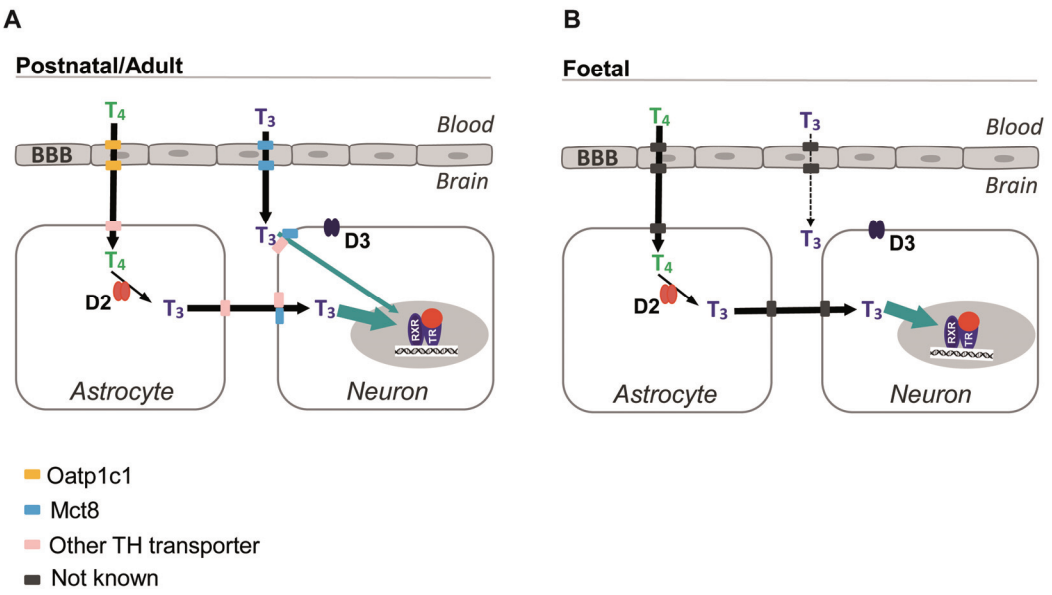


Figure 3. Sources of T3 in the postnatal and foetal brain. (A) In the adult brain, T3 from the circulation crosses the BBB through Mct8 transporter and directly access the neurons in the brain where it binds to the TR– RXR heterodimer and regulate gene expression. Also, T4 from the circulation can cross the BBB through Oatp1c1 transporter and reach the astrocytes where is converted into T3 by D2 activity. Passage of T3 from astrocytes to neurons is then facilitated by Mct8 and also other transporters. (B) In the foetal brain the main source of T3 is through deiodination of T4. The contribution of TH transporters to T3 brain availability during the foetal period is still not well understood. *Modified from Morte and Bernal, (2014).*

50% of the total brain T3 derives directly from T3 uptake from the circulation and another 50% is locally generated in the astrocytes.

On the contrary, during foetal development the brain depends almost entirely on the T3 locally generated by D2 in the astrocytes as has been demonstrated in studies in rats (**Figure 3 B**). Administration of high doses of T3 to hypothyroid pregnant rats were able to cross the placenta but failed to normalise T3 content in the foetal brain and to induce the expression of T3-dependent genes. In contrast, physiological doses of T4 could cross both the placenta and the foetal brain barriers normalising foetal brain T3 concentrations by T4 deiodination and increasing neuronal gene expression (Calvo et al., 1990, Grijota-Martínez et al., 2011). The role of D2 activity in this process is further supported by D2 activity ontogeny studies that revealed a significant D2 activity in rat foetal brain that highly increased prior to birth and that was inversely regulated by increasing doses of T4 (Ruiz de Oña et al., 1988). The reason why the foetal brain is not permeable to T3 is not known and cannot be explained by lack of the Mct8 transporter as it is present at the BBB during foetal development (Grijota-Martínez et al., 2011).

2.3 Ontogeny of thyroid hormone action in rat brain

The rat as an animal model has provided most of the current knowledge about thyroid physiology and TH action in brain. In the rat, embryonic tissues are provided with T4 and T3 from at least embryonic day 11 (E11) (Obregon et al., 1984) which is well before the onset of foetal thyroid gland function, that in the rat starts after E17. As the foetal thyroid gland starts functioning, the proportion of foetal TH increases while the maternal decreases and at term about 17.5% of the foetal extra thyroidal T4 pool is of maternal origin (Morreale de Escobar et al., 1990).

The few studies reporting T3 and T4 content in the developing rat brain have been restricted to periods coinciding or following the onset of foetal thyroid function at E17 - E21 (Morreale de Escobar et al., 1985), however, there is evidence that maternal TH is necessary before the onset of the foetal thyroid function as maternal hypothyroidism leads to defects in the proliferation of some neuronal precursors that is usually completed by E12 (Narayanan and Narayanan, 1985) or to defects in the migration of some proliferating cells that normally finishes at E16-E17 (Lucio et al., 1997). mRNA for TRs has been detected from E11.5 at the neural tube and from E12.5 in certain areas of the prosencephalon, mesencephalon and the rhombencephalon (Bradley et al., 1992). In addition, T3 binding assays in brain have revealed the presence of TRs bound to T3 from E14 with the concentration of binding sites increasing progressively until E17 and remaining until birth (Perez-Castillo et al., 1985). In the rat, deiodinase activity assays have detected increasing D2 activity in the brain during development from as early as E17 until the day prior to birth (Ruiz de Oña et al., 1991) (**Figure 4**). Therefore, TH could exert an action in restricted brain regions or groups of cells during early stages of development and have a clear role during late gestation.

Furthermore, the contribution of maternal TH to foetal development under euthyroid conditions still remains unclear. One study revealed that foetuses coming from thyroidectomised pregnant rats presented only a small decrease in the brain content of T4, with no changes in T3 content at E21 so the gene expression of T3-dependent genes was accordingly unaffected in comparison to foetuses coming from control pregnant dams (Grijota-Martínez et al., 2011). Likewise, in another study, brain D2 activity of foetuses coming from thyroidectomised pregnant rats was not different from those coming from control dams (Ruiz de Oña et al., 1988). These findings can be attributed to the progressive activity of the foetal thyroid gland.

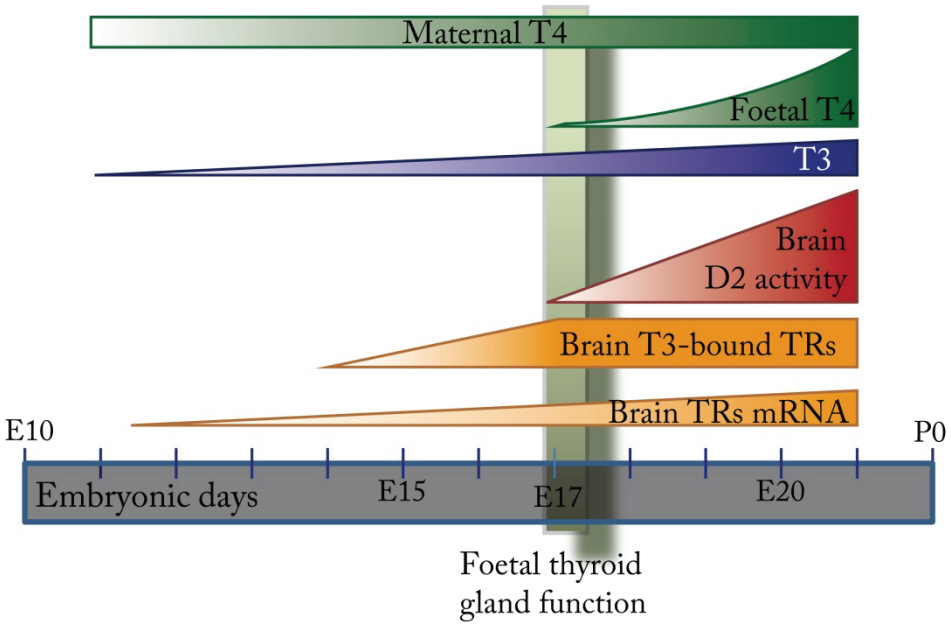


Figure 4. Ontogenesis of thyroid hormone action during foetal development in the rat. In the rat, before the onset of foetal thyroid function at E17, embryonic tissues are already provided with T4 and T3 from E11. The proportion of foetal T4 gradually increases after the foetal thyroid gland starts functioning. In the brain, TRs mRNA expression from E11.5 and T3-bound TRs from E14 suggest an early role for thyroid hormone action. D2 activity as well as T4 and T3 content are present in the foetal brain from at least E17. Dimensions of the figures do not represent relative abundance.

Nevertheless, it seems clear that deiodination of T4 (whether maternal or foetal) is the major source of T3 to the foetal brain (Escobar-Morreale et al., 1999), at least during late gestation, and also that in foetal hypothyroid conditions, maternal T4 can protect the foetal brain (Grijota-Martínez et al., 2011, Calvo et al., 1990).

Due to the advantages of using genetic engineering approaches to generate genetically modified animals in mice over rat, the mouse has lately become the most widely used model to study thyroid physiology and TH action in brain. The use of genetically modified mice is providing new insights about thyroid physiological and pathological events. Considering the essential role that TH play during brain development it is crucial to explore TH brain availability during mouse brain development in order to understand both developmental and latter events. To date this has not been extensively studied probably due to technical difficulties in the manipulation of mouse perinatal brain such as small amounts of tissue, difficulties to process the delicate tissues from these stages without damage or distortion or the very specific spatiotemporal working frames. There is also the mistaken tendency to assume that mouse embryonic development follows the same pattern as rat. It is therefore essential to

clarify some key aspects regarding TH availability and economy in the brain during mouse foetal development.

2.4 Thyroid hormone and brain development

TH are essential for the correct development of the CNS by regulating processes such us neurogenesis and glial cell differentiation including neuronal migration, myelination, and synaptogenesis in a very specific time window (Bernal, 2015). Much of the current knowledge about the actions of TH in brain development arises from indirect evidence obtained by studying the consequences of TH deficiency during critical stages of development. Recent transcriptomic analysis from our group has revealed a broad role for TH, suggesting that T3 promotes the transition of gene expression from foetal to adult patterns. T3 induces the expression of genes related to processes such as synaptic transmission and neurotransmission, chemical homeostasis or ion transport and inhibits expression of genes involved in cell division, chromosome segregation and organization during the M phase of cell cycle (Gil-Ibañez et al., 2015).

Lack of sufficient TH results in abnormal development and has different functional consequences depending on the timing and the cause for TH deficiency. Due to the importance of maternal TH contribution to foetal development it is important to define maternal hypothyroidism and maternal hypothyroxinemia. The American Thyroid Association defines primary maternal hypothyroidism as the presence of elevated TSH concentrations with decreased FT4 concentrations for overt hypothyroidism or with normal FT4 concentration for subclinical hypothyroidism. Isolated hypothyroxinemia is defined as normal maternal TSH concentration in conjunction with FT4 concentrations in the lower 5th or 10th percentile of the reference range (Stagnaro-Green et al., 2011).

- ### Iodine Deficiency Disorders. Endemic Cretinism

Iodine deficiency during embryonic development leads to a syndrome known as cretinism that manifests as two main forms, which today are considered the extremes of the same condition. Historically these two forms were known as neurological or myxedematous. In neurological cretinism the thyroid gland appears to be normal and patients are euthyroid, however, they present severe mental retardation, bilateral hearing loss, spasticity affecting specially the lower limbs, rigidity and bradykinesia (DeLong et al., 1985). In myxedematous cretinism patients suffer from hypothyroidsm and thyroid atrophy and as a result they present immature sexual

development, short stature, craniofacial abnormalities and in some cases mental retardation, although not as severe as in neurological cretinism (Boyages et al., 1988). The reason for these different phenotypes is a differential timing in the action (or lack of action) of TH. The clinical features characteristic of neurological cretinism suggests lack of TH action during the second trimester of pregnancy due to iodine deficiency that causes iodine deprivation to the foetus and severe maternal hypothyroxinemia during the first half of pregnancy. However, myxedematous cretinism is due to a non-functional foetal and infant thyroid gland during the last trimester of pregnancy and the postnatal period. Very often the destruction of the foetal gland is as consequence of iodine deficiency and goitrogens in the diet or lack of selenium (Bernal, 2015). Both forms of cretinism often coexist in the same population.

- **Congenital Hypothyroidism**

Congenital hypothyroidism results from developmental defects in the thyroid gland itself or from impaired synthesis of TH. If not treated shortly after birth, patients present severe mental retardation, short stature, constipation, and other hypothyroid symptoms and signs. As the success of treatment with TH depends on the severity, onset, and duration of hypothyroidism, systematic screening for congenital hypothyroidism in the newborn is essential to prevent mental retardation (Krude et al., 2015).

- **Maternal Hypothyroidism and Hypothyroxinemia**

Maternal TH cross the placental barrier and may play a role during embryonic development before and after the onset of the foetal thyroid function (Morreale de Escobar et al., 1987). There is a considerable transfer of maternal T4 to the foetus near term which can be crucial to protect the foetal brain in cases of congenital hypothyroidism (Vulsma et al., 1989). Both, maternal hypothyroidism and maternal hypothyroxinemia are associated with reduced intelligence quotient (IQ) in children, and increased incidence of Attention Deficit Hyperactivity Disorder (Bernal, 2015). To avoid maternal hypothyroxinemia, and to provide enough iodine to the foetus, iodine supplementation for women considering conception and during pregnancy and lactation is recommended (Morreale de Escobar et al., 2004, Berbel et al., 2007).

- **Resistance to thyroid hormone syndromes**

Resistance to thyroid hormone (RTH) syndromes are a group of genetic conditions that lead to decreased tissue sensitivity to TH. These include mutations in TH receptors, TH transporters, and impaired deiodinase activities (Dumitrescu and Refetoff, 2015).

T3 receptor mutations include mutations in the *THRB* and *THRA* gene. Patients with mutations in TRβ1 and TRβ2 commonly present reduced IQ, learning disabilities, hyperactive behaviour, goitre, tachycardia and bone mineralization disorders. Elevated serum TH levels with non-suppressed TSH usually leads to the diagnosis, which is confirmed by *THRB* sequencing (Takeda et al., 1992). Patients with mutations in the *THRA* gene were not described until 2012 and they present growth and developmental delays, skeletal dysplasia, and severe constipation. In the serum, they present relatively low T4 and slightly elevated T3 levels, so in this case, there is an elevated T3/T4 ratio (Bochukova et al., 2012). Diagnosis is confirmed by *THRA* sequencing.

Mutations in the TH transporter MCT8 lead to the Allan-Herndon-Dudley Syndrome (AHDS) and will be described in detail below.

Up to now no inactivating mutations in the *DIO2* gene in humans have been reported. The only inherited defect of intracellular TH metabolism described so far is caused by mutations in the selenocysteine insertion sequence binding protein 2 (SBP2) which is a transacting factor involved in the co-translational insertion of selenocysteine into selenoproteins (Driscoll and Copeland, 2003). The clinical phenotype consists on short stature, delayed bone age and unusual thyroid function test abnormalities with high T4, low T3, and normal or slightly elevated TSH serum levels (Dumitrescu and Refetoff, 2011).

3. Mutations in the MCT8 transporter

MCT8 is the only TH transporter with known pathogenic mutations in humans that lead to the AHDS.

3.1 MCT8

Monocarboxylate transporter 8 (MCT8) is the protein product of the solute carrier family 16 member 2 gene (*SLC16A2* or *MCT8*) and it is localised in the q13.2 region of the X chromosome (Lafrenière et al., 1994). The gene consists of 6 exons and, in humans, there are two translation start sites generating two isoforms with different amino acid lengths of 613 or 539. Mouse *Mct8* only generates a 545 amino acid isoform, which is shorter at the N- terminus. In both species, the gene codes for a protein of between 58 and 66 kDa with 12 transmembrane domains, characteristic of transporter proteins, with the N- and C-terminus located intracellularly. At the N-terminal end there is a PEST domain that serves as proteolytic signal,

targeting the protein for rapid degradation (Lafrenière et al., 1994). It has recently been demonstrated that the long human MCT8 isoform contains a longer N-terminus which enhances ubiquitin-dependent proteasomal degradation affecting the amount of MCT8 protein (Zwanziger et al., 2016). MCT8 exists as monomer and homodimer and in humans, MCT8 dimers are more stable as this complex is more resistant to denaturing conditions (Visser et al., 2009).

Mct8 is present in the brain, heart, placenta, lung, kidney, skeletal muscle, and, most strongly, in the liver (Friesema et al., 2005). In the brain Mct8 is expressed in astrocytes, neurons, oligodendrocyte precursor cells and at the BBB (Bernal et al., 2015) and the choroid plexus (Roberts et al., 2008).

3.2 Etiology and clinical manifestations

In 1944 Allan and colleagues described a sex-linked form of mental retardation with motor abnormalities and named it the AHDS (Allan et al., 1944). The syndrome was mapped to a locus on chromosome X in 1990 (Schwartz et al., 1990) but it was not until 2004 that mutations in the gene encoding for the TH-specific cell membrane transporter MCT8, were identified to result in the AHDS in males (Dumitrescu et al., 2004, Friesema et al., 2004).

MCT8 deficient patients appear normal at birth and the syndrome starts to become evident in the first few months showing global developmental delay, severe intellectual disability, lack of speech, profound neuromotor impairment with central hypotonia that progresses to spastic quadriplegia, dystonic movements, and muscle wasting. As well as the neurological component, the syndrome also consists of an endocrine component as patients present altered serum concentration of TH with elevated T3, low T4 and rT3, and normal or slightly elevated TSH, indicating a defect in TH metabolism (Dumitrescu et al., 2004, Friesema et al., 2004).

Evaluation of thyroid status, including both T3 and T4, as TSH levels might not be affected, can guide the diagnosis until genetic confirmation. Since the first descriptions of mutations in MCT8 in 2004 more than 100 families in the world have been diagnosed with the syndrome. In Spain, the generation of a MCT8 deficiency clinical guide by Dr Juan Bernal and Dr Beatriz Morte allowed for the identification and description of the first patient in 2013 (Lopez-Marin et al., 2013) and since then, 4 other cases have been reported. This highlights the importance of both being aware of and suspecting this syndrome in order to obtain an early diagnosis.

3.3 Treatment

The therapeutic options for these patients are rather limited. In an initial attempt, since patients present low serum T4 levels, treatment with levothyroxine (LT4) was tested with no improvement in the neurological function and even further increasing the serum T3 levels that worsened the peripheral hyperthyroidism (Biebermann et al., 2005, Zung et al., 2011). To avoid the latter, propylthiouracil (PTU) has been administered together with LT4. This treatment allows T3 levels to be normalised in peripheral tissues, improving the thyrotoxic state, but it does not improve the neurological condition (Visser et al., 2013, Wemeau et al., 2008).

In another approach, four young MCT8-deficient patients were treated with the TH analogue 3,5-diiodothyropropionic acid (DITPA) which is an agonist of the T3 nuclear receptor. This treatment normalised the serum TH levels, ameliorating the thyrotoxicosis of peripheral tissues but unfortunately no improvements in psychomotor development were observed (Verge et al., 2012).

Recently and during the course of this thesis, another TH analogue, 3,3',5-triiodothyroacetic acid (TRIAC), is being considered as an alternative treatment and a clinical trial is being developed exploring as a first goal its action at the peripheral level. The outcome of this clinical trial so far will be discussed along with some of the results obtained in this thesis in the discussion section.

In summary, the therapeutic strategies currently available are successful in alleviating the thyrotoxicosis of peripheral tissues by normalising the circulating levels of T3, but are not able to improve the neurological impairments of MCT8 deficient patients.

3.4 Mct8 deficient mice

Mct8 knockout (*Mct8*KO) mice were generated as a model for the AHDS. These mice faithfully replicate the alterations in the TH concentrations in serum of the patients with high T3 and low T4 (Dumitrescu et al., 2006, Trajkovic et al., 2007). Unfortunately, they do not present gross neurological abnormalities (Wirth et al., 2009), hence they are only a partial model of the disease. Despite of this, they have been a useful tool to understand some aspects of the syndrome and to explore the differences between mice and humans. Studies using these mice have demonstrated that despite having high serum T3 concentrations, *Mct8*KO mice have

reduced T3 content in the brain (Dumitrescu et al., 2006, Trajkovic et al., 2007) and alterations in the expression of some T3-dependent genes (Morte et al., 2010).

Studies with *Mct8*KO mice have also suggested that the pathogenesis associated with MCT8 deficiency arises from an impaired T3 transport across the BBB. In these studies, when hypothyroid *Mct8*KO animals were treated with T4 or T3, T4 was considerably more successful in normalising the expression of T3-dependent genes indicating a restricted access of T3, but not T4, to neurons. However, T3 action in neurons *in vitro* was similar in Wild type (Wt) and *Mct8*KO cells suggesting that the main restriction for T3 entry into the neural target cells of *Mct8*KO mice is at the BBB (Ceballos et al., 2009) and not at the neuronal membrane as previously believed. Therefore, in the absence of Mct8, T3 cannot enter in sufficient amounts to the brain. This has been supported by recent studies of human brain sections that have provided evidence for cerebral hypothyroidism in MCT8 deficient patients, already present from prenatal stages (López-Espíndola et al., 2014).

The current hypothesis that explains why *Mct8*KO mice do not present gross neurological abnormalities in contrast to the severe neurological impairments of MCT8 deficient subjects supports that the elevated D2 activity, characteristic of *Mct8*KO mice in the brain (Dumitrescu et al., 2006, Trajkovic et al., 2007), enhances local T4 to T3 conversion as a compensatory mechanism (**Figure 5**). It has been proposed that T4 crosses the BBB and/or the BCSFB in the absence of Mct8 through an additional transporter. The Oatp1c1 transporter was proposed as a candidate to mediate T4 transfer into the brain as it is predominantly localised in capillary endothelial cells as well as in choroid plexus structures (Pizzagalli et al., 2002, Sugiyama et al., 2003) and most importantly, OATP1C1 expression has not been detected in BBB endothelial cells of primate brain (Ito et al., 2011). This hypothesis was supported by the generation of double knockout (KO) animals lacking both Mct8 and D2 proteins or Mct8 and Oatp1c1 transporters as both animal models present similar characteristics to hypothyroidism (Morte et al., 2010, Mayerl et al., 2014). In addition, the phenotypic outcome of these double knockouts further validated the model for TH brain availability described above.

In summary, Mct8 deficiency in adult mice leads to peripheral hyperthyroidism with T3 excess (in the plasma, liver, kidney and muscle) due to complex mechanisms that are not fully understood and are summarised in **figure 5**, and brain hypothyroidism due to T3 deprivation (**Figure 5**).

Surprisingly, at perinatal stages of development *Mct8*KO mice exhibit a transient state of brain hyperthyroidism with increased brain T4 and T3 content and increased expression of T3-

dependent genes (Ferrara et al., 2013). The mechanisms underlying this brain hyperthyroidism are not well understood but it has been suggested that could be either a result of increased local production of T3 from T4, or to retention of T3 in the cortex. This last one could indicate that Mct8 mediates T3 efflux and/or degradation of T3 by D3 in neurons (Nuñez et al., 2014).

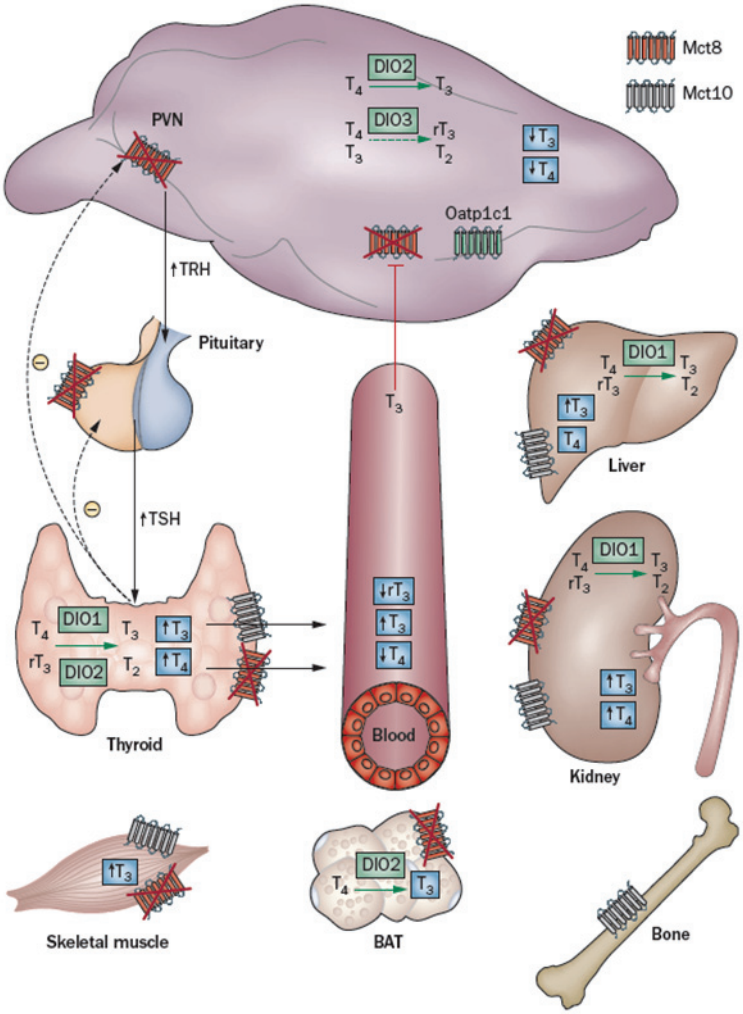


Figure 5. Changes in TH concentration in Mct8 deficient (*Mct8*KO) mice. In the plasma, they present high T3 and low T4 and rT3. In the absence of Mct8, there is an impaired regulation of the hypothalamic–pituitary axis as T3 cannot reach the paraventricular nucleus (PVN) of the hypothalamus and the pituitary, leading to resistance of the TRH–TSH axis to TH. In the brain, deficient T3 entry reduces deiodination by D3 contributing to the increased levels of T3 in serum. Although T4 entrance is reduced in the brain, transport through the Oatp1c1 transporter allows the conversion of T4 to T3 by increased D2 activity. In the liver, increased supply of T3 from the plasma produces liver hyperthyroidism which increases D1 activity producing even more T3 from T4. In the kidney, Mct8 deficiency decreases efflux with retention of T4 and T3 producing kidney hyperthyroidism that increases D1 activity as in liver. In bone, an alternative transporter (Mct10) might mediate T3 transport and action. In brown adipose tissue (BAT), reduced T4 levels increase D2 activity, which results in normal T3 concentrations and BAT function. In skeletal muscle, the increased T3 content leads to an increase in the energy expenditure. *From Bernal et al., (2015).*

OBJECTIVES

The broad aim of the present study is to explore TH availability and action in mouse brain during foetal development in health and disease. Understanding how TH accesses and functions in the developing brain, among other things, might allow the design of therapeutics and timing of treatments for pathological conditions related to TH, such as for the AHDS. Moreover, in the absence of an effective therapy for MCT8 deficient patients, this study has aimed to develop treatments for MCT8 deficiency according to the current knowledge. It has therefore been divided into two separate parts.

1. The first part has aimed to explore the sources of T3 that ensure brain euthyroidsm during mice foetal development focusing on:
   - the contribution of maternal TH to foetal development,
   - the contribution of TH transporters and deiodinases activities in the foetal brain with special attention to D2 activity due to its relevance in the postnatal and adult mouse and
   - unravelling the role of D2 in Mct8 deficiency during early developmental stages.

2. The second part has aimed to test novel therapeutic approaches as possible treatments for MCT8 deficiency using *Mct8*KO mice. Taking into account that the main restriction for T3 brain entry in MCT8 deficiency is at the BBB, these strategies have been:
   - the use of the TH analogue TRIAC that is not transported by Mct8 and that has the ability to cross the brain barriers,
   - the replacement of MCT8 by adeno-associated virus to try to correct brain defects and
   - the administration of TH replacement treatments by intranasal administration, a delivery route that can circumvent the BBB.

MATERIALS AND METHODS

1. Ethics statement

All experimental procedures involving animals were performed following the European Union Council guidelines (directive 2010/63/UE) and Spanish regulations (R.D. 53/2013), and were approved by the ethics committee at Consejo Superior de Investigaciones Científicas (CSIC; approval numbers 315/14 and 316/14). All efforts were made to minimise suffering, as indicated below.

2. Experimental animals

Animals were housed in temperature- and light-controlled conditions at 22 ± 2° C on a 12:12 light-dark cycle (lights on at 8 am), and they had access to food and water *ad libitum*.

Mct8 (*Slc16a2*) KO mice (male genotype, *Mct8*$^{-/y}$) were originally produced by Dr. Refetoff (Dumitrescu et al., 2006) by homologous recombination. Experiments were carried out in *Mct8*$^{+/y}$ and *Mct8*$^{-/y}$ mice from the same litters derived by backcrossing heterozygous females with Wt males of the C57BL/6J strain at our animal facility. The pups were genotyped to select for *Mct8*$^{+/y}$ and *Mct8*$^{-/y}$ mice from the same litters. For simplicity, these animals will be referred to as Wt and *Mct8*KO mice, respectively. The *Mct8* genotype was confirmed by PCR of tail DNA (35 cycles at 61° C annealing temperature) using the following primers: forward common, 5′-ACAACAAAAAGCCAAGCATT-3′; reverse Wt specific, 3′-GAGAGCAGCGTAAGGACAAA-5′; reverse *Mct8*KO specific, 3′-CTCCCA AGCCTGATTTCTAT-5′ which generates a 476-bp product corresponding to the Wt allele and a 239-bp PCR product corresponding to the null allele.

*Dio2*KO (*Dio2*$^{-/-}$) mice and Wt counterparts were initially provided by Dr. Galton (Schneider et al., 2001) and a colony was established at Instituto de Investigaciones Biomédicas (Madrid, Spain) in the same C57BL/6J genetic background. The *Dio2* genotype was confirmed by PCR of tail DNA (35 cycles at 62° C annealing temperature) using the following primers: forward common, 5′-GTTTAGTCATGGAAGCAGCACTATG-3′; reverse Wt specific, 5′-CATGGCGTTAGCCAAAACTCATC -3′ and reverse *Dio2*KO specific, 5′-CGTGGGATCATTGTTTTTCTCT-3′ which generates a 400-bp product corresponding to the Wt allele and a 450-bp PCR product corresponding to the null allele.

Double Mct8- and D2-deficient mice (*Mct8*/*Dio2*KO; *Mct8*$^{-/y}$ and *Dio2*$^{-/-}$) were generated in the same C57BL/6J genetic background by mating *Mct8*$^{-/y}$ and *Dio2*$^{-/-}$ mice. The *Mct8*$^{-/y}$ *Dio2*$^{-/-}$ was confirmed by the PCRs procedures described above.

3. Treatments

3.1 Thyroid hormone treatment

After crossing with Wt male mice, Wt pregnant dams were given either T3 or T4 in the drinking water from E12 until the end of the experiment on E18, the day prior to birth. The TH concentration was calculated on the basis of fluid intake to provide 1.5 µg T3/100 g body weight (BW) or 8 µg T4/100 g BW in the drinking water containing 0.01% bovine serum albumin (BSA) and was not corrected for increasing weight. To analyse the relative effects of the maternal and the foetal thyroidal status, the following groups were compared: control euthyroid dams receiving no hormonal treatment (n = 7); dams treated with 1.5 µg T3/100 g BW from E12 (n = 6) and dams treated with 8 µg T4/100 g BW from E12 (n = 7). On gestational day E18 dams were killed in a CO_2 euthanasia chamber and were perfused with saline to remove blood from tissues before their collection. Prior to perfusion, blood was extracted by retroorbital collection and used for the determination of T4 and T3 plasma concentrations. Embryos were killed by decapitation at E18 and their brains and livers were dissected out, blotted on filter paper, frozen on dry ice, and stored at -80° C.

3.2 TRIAC treatment

Wt and *Mct8*KO mice from the same litters were given either drinking water or TRIAC (Sigma-Aldrich) in the drinking water at P21, concurring with the weaning, until P30. The TRIAC concentration was calculated on the basis of fluid intake to provide 30 ng/g BW/day in the drinking water containing 0.01% BSA. Animals were killed at P30 two hours after the end of the dark cycle, when most of the water intake takes place.

In one set of experiments, the following groups were prepared: Wt mice (n = 5), *Mct8*KO mice (n = 7), Wt mice treated with TRIAC (n = 5), and *Mct8*KO mice treated with TRIAC (n = 7). The animals were killed by decapitation. Trunk blood was collected in heparinized tubes after

decapitation and was used for T3, T4, and TRIAC determinations in plasma. The cerebral cortex, striatum, liver and heart were harvested as explained below. These tissues were used for gene expression analysis.

In another set of experiments, Wt mice (n = 7), *Mct8*KO mice (n = 10), Wt mice treated with TRIAC (n = 8), and *Mct8*KO mice treated with TRIAC (n = 11) were used to determine the T3 and TRIAC content in the cerebral cortex and the striatum.

3.3 Intranasal treatment

Intranasal administration was performed as described in Dhuria (Dhuria et al., 2010). Mice where anaesthetised with isoflurane (4% for induction, 2% for maintenance in O_2, Abbot Laboratories, Madrid, Spain). After induction of anaesthesia, mice were placed lying on their backs maintaining their head positioned so drainage of the dose solution into the trachea and oesophagus was prevented. Because of the protective barriers in the nasal mucosa, typically less than 1% of the administered dose reaches the brain so a highly concentrated drug formulation is required for intranasal administration.

In one set of exploratory experiments a high concentrated solution of T4 was intranasally administered to three-month-old mice. Mice were euthalized after a long-term treatment (Wt basal n = 2, intranasally treated with saline; Wt + T4 n = 3, intranasally treated with T4; *Mct8*KO basal n = 3, intranasally treated with saline and *Mct8*KO + T4 n = 5, intranasally treated with T4) or after a short-term treatment (Wt basal n = 3; Wt+T4 n = 3; *Mct8*KO basal n = 2 and *Mct8*KO +T4 n = 5).

In another set of experiments a high concentrated solution of T3 was intranasally administered to Wt mice. T3 was administered alone, bound to 1% BSA or bound to 1% BSA in the presence of a vasoconstrictor, phenylephrine hydrochloride (PHE; Sigma, P6126). The following groups were prepared: 3-month-old Wt basal n = 11 (Wt treated with saline intranasally delivered); Wt + T3 n = 11 (Wt treated with T3 intranasally delivered); Wt + T3-BSA n = 7 (Wt treated with T3 bound to 1% BSA intranasally delivered) and Wt+T3-BSA-PHE n = 7 (Wt treated with T3 bound to 1% BSA and 1% PHE intranasally delivered). Mice were sacrificed after a short-term treatment and brains and plasma were harvested for posterior analysis.

4. Tissue extraction and processing

Tissue employed for TH determinations, D1 and D2 activity assays as well as for RNA extraction of juvenile or adult mice was obtained by anesthetising the animals with ketamine (75 µg/g BW) and medetomidine hydrochloride (1 µg/g BW), and perfused with saline to remove blood from tissues before their collection. Prior to perfusion, blood was extracted by retroorbital collection and used for TH determinations. Brains were removed, and the cerebral cortex and the striatum were rapidly dissected out from the underlying structures. The liver and heart were also harvested.

Wt (n = 14), *Mct8*KO (n = 14) littermates; Wt (n = 8), *Dio2*KO (n = 8) littermates; and *Dio2*KO (n = 8), *Mct8/Dio2*KO (n = 10) littermates at P3 were euthanized by decapitation and the cerebral cortices were dissected at 4° C in PBS (phosphate-buffered saline: phosphate buffer 0.05 M and 0.9% NaCl, pH 7.4). All tissue was blotted on filter paper, frozen on dry ice, and stored at -80° C.

Tissue employed for histological procedures of juvenile or adult mice was obtained by anesthetising the animals with ketamine (75 µg/g BW) and medetomidine hydrochloride (1 µg/g BW) and perfusing with saline followed by 4% paraformaldehyde in 0.1 M PB (phosphate buffer 0.1M, pH 7.4) via injection into the left heart ventricle. Brains were then removed and postfixed for 24 h and afterwards embedded in paraffin. Perinatal animals (E15, E18, P1, P3) were euthanized by decapitation; the brains were removed and fixed in 4 % paraformaldehyde in 0.1 M PB for 24 h and afterwards embedded in paraffin. A minimum of two animals per group was used for histological studies. Paraffin blocks containing cerebral cortex were cut coronally while those containing cerebellum were cut sagitally into 8 µm thick sections that were transferred onto Superfrost Ultra Plus slides (Thermo Scientific).

5. Radioimmunoassays of T4, T3, and TRIAC in plasma and tissues

T3, T4 and TRIAC determinations were performed in collaboration with Dr. Maria Jesus Obregon of the Instituto de Investigaciones Biomédicas “Alberto Sols” in Madrid.

T3, T4 and TRIAC were labelled with ^{125}I from T2, T3 and DIAC respectively with a high specific activity (3,000-1,500 µCi/µg) according to Weeke and Orskov (1973). Products of the synthesis were separated by paper chromatography in butanol-methanol-ammonia-water as described (Obregon et al., 1978).

For postnatal animals, individual 80 µl aliquots of plasma as well as hemicortices and striatum of individual mice were extracted for radioimmunoassay (RIA) determinations. For E18 mice, hemicerebrums and livers from individual embryos were extracted for RIA determinations. Extraction of T3, T4 and TRIAC from the plasma and tissue as well as T3, T4 and TRIAC determinations in plasma and tissues, were determined as previously described (Morreale de Escobar et al., 1985, Medina-Gomez et al., 2008). When animals had been treated with TRIAC, T3 data were corrected for the crossreactivity of TRIAC on the T3 antiserum (17%).

6. D1 and D2 enzymatic activities

D1 and D2 enzymatic activities were performed in collaboration with Dr. Maria Jesus Obregon of the Instituto de Investigaciones Biomédicas "Alberto Sols" in Madrid.

D1 activity in liver and D2 activity in the hemicortices and hemicerebrums were measured as described (Obregon et al., 1989) with minor modifications. In brief, the release of ^{125}I iodide from ^{125}I-labeled substrates was measured in tissue homogenates using 0.32 M sucrose, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and 10 mM dithiothreitol (DTT). ^{125}I-labelled substrates were purified by removing contaminating iodide by electrophoresis.

For D2 activity in hemicortex and hemibrain: ^{125}I-T4 (100,000 – 50,000 cpm/tube), 2 nM T4 + 1 µM T3, 20 mM DTT, and 1 mM PTU per 100 µl were incubated for 60 min at 37° C using 20-50 µg protein/100 µl.

For D1 activity in liver: ^{125}I- rT3 (60,000 cpm/tube) was used in the presence of 200 nm rT3 and 2 mm DTT, and were incubated for 10 min at 37° C, using 20–50 µg protein/100 µl.

The ^{125}I iodide released was directly measured in a gamma counter detector. The protein content was determined by the method of Lowry et al. (1951) after precipitation of the homogenates with 10% trichloroacetic acid to avoid interference from DTT in the colorimetric reaction.

7. Gene expression

RNA was isolated from individual hemicerebrums, striatum, hemicerebral cortices, liver and heart. Total RNA was extracted using TRIZOL reagent (Invitrogen, 15596026) following the manufacture's recommendations with an additional chloroform extraction. RNA quality control was performed with the Agilent 2100 Bioanalyzer. cDNA was prepared from 250 ng RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). For quantitative PCR (qPCR), a cDNA aliquot corresponding to 5 ng of the starting RNA was used. The real time PCR was performed with the Taqman universal PCR master mix, No Amp Erase UNG (Applied Biosystems), on a 7900HT fast real-time PCR system (Applied Biosystems). The PCR program consisted in a hot start of 95° C for 10 min, followed by 40 cycles of 15 sec at 95° C and 1 min at 60° C. PCRs were performed in triplicates, using the 18S gene as internal standard and the 2-cycle threshold method for analysis. Data were expressed relative to the values obtained on tissues from the Wt mice in basal conditions, which were given a mean value of 1.0 after correction for 18S RNA.

The Taqman probes (Applied Biosystems) used in this thesis are listed below:

Probe ID	Gene symbol	Full gene name
Mm00657317_m1	Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1
Mm00483074_g1	Cbr2	carbonyl reductase 2
Mm00839358_m1	Dio1	deiodinase type 1
Mm00515664_m1	Dio2	deiodinase type 2
Mm00548953_s1	Dio3	deiodinase type 3
Mm00513052_m1	Flywch2	flywch family member 2
Mm00498963_m1	Hr	hairless
Mm00434548_m1	Itih3	inter-alpha trypsin inhibitor, heavy chain 3
Mm00495172_m1	Klf9	kruppel-like factor 9
Mm00441516_m1	Lat1, Slc7a5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
Mm00444250_m1	Lat2, Slc7a8	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
Mm00486204_m1	Mct8, Slc16a2	solute carrier family 16 (monocarboxylic acid transporters), member 2
Mm00661045_m1	Mct10, Slc16a10	solute carrier family 16 (monocarboxylic acid transporters), member 10
Mm00453126_m1	Oatp1a4, Slco1a4	solute carrier organic anion transporter family, member 1a4
Mm00451845_m1	Oatp1c1, Slco1c1	solute carrier organic anion transporter family, member 1c1
Mm01201431_m1	Serca2a, Atp2a2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2
Mm00436528_m1	Shh	sonic hedgehog

8. BSA-T3 binding assay

This was performed to evaluate the lowest concentration of BSA necessary to bind the appropriate concentration of T3 required for intranasal delivery.

The ability of BSA to bind a T3 replacement dose (Di Cosmo et al., 2009) was also assessed. These solutions were incubated for 20 min at room temperature (RT) with 18,000 cpm of ^{125}I-T3 and in presence of different concentrations of BSA: 0.25%, 0.5%, 1%, 1.5%, 2% and 5%. Prior to incubation ^{125}I-T3 was purified by removing contaminating iodide by electrophoresis. After incubation samples were precipitated with 10% trichloroacetic acid by centrifuging 30 min at 2,800 rpm and the supernatants were removed. The ^{125}I-T3 bound to BSA precipitated was assessed by measuring the ^{125}I in a gamma counter detector. Percentage of binding was calculated by referring these measurements to standard samples solely containing the 18,000 cpm of ^{125}I-T3.

9. Immunohistochemical and immunofluorescence procedures

Tissue sections were deparaffinised by incubating the sections 20 min at 65° C prior to three incubations with xylenes at RT and then washed in serial dilutions of ethanol in order to rehydrate the tissues. Next, tissue sections were incubated 20 min at 95° C with the EnVision FLEX Low pH (Dako, K8005) solution for antigen retrieval.

For diaminobenzidine labelling, endogenous peroxidase was blocked at RT for 15 min with 3% hydrogen peroxide in distilled water. Afterwards, nonspecific antibody binding was prevented by blocking the tissue in PBS containing 0.1% triton X-100, 4% BSA (Sigma, A4503) and 5% normal goat serum (Vector Laboratories, S-1000) at RT for 1.5 h. Tissue sections were incubated at 4° C overnight with the primary rabbit anti-hMCT8 1:250 (Sigma, HPA029308) in PBS containing 0.1% Triton X-100, 4% BSA and 1% normal goat serum. After 6 washes with PBS, sections were incubated for 1 h at RT with biotinylated anti-rabbit secondary antibodies (Vector Laboratories, BA-1000) at a 1:200 dilution in PBS containing 0.1% Triton, 4% BSA and 1% normal goat serum. For signal amplification the sections were incubated with Avidin-Biotin Complex (Thermo Scientific; Ultra-Sensitive ABC Peroxidase Staining Kits, 32050) at RT for 1 h and revealed with diaminobenzidine (5mg/mL, Sigma, D5637). Some preparations were counterstained with Harris hematoxylin (Sigma, HHS32). Finally, tissues were dehydrated in ascending alcohols, cleared in xylenes, and covered with

hydrophobic mounting medium Depex (Serva, 18243). Moreover, in order to verify the specificity of the secondary antibody, negative controls were run in parallel without the primary antibody. Histological analyses were made under bright field illumination using a Nikon Eclipse 80i microscope and photomicrographs were acquired with a Nikon DS-Fi1 digital camera.

For immunofluorescence labelling, antigen retrieval, nonspecific binding prevention and incubation with primary rabbit anti-hMCT8 were performed as described above with the difference that this time the anti-hMCT8 was used at 1:200. The secondary antibody Goat anti-rabbit Alexa 546 (Molecular Probes, A-11035) was used at a 1:500 dilution in PBS containing 0.1% Triton, 4% BSA and 1% normal goat serum at RT for 2 h. The sections were posteriorly washed 6 times in PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, D1306) 1:500 in PBS. After washing 3 times in PBS, sections were mounted with ProLong Gold Antifade Mountant (Molecular Probes, P36930). Omitting the primary antibodies in the incubation reaction gave no signal. Confocal images were acquired using an inverted Zeiss LSM 710 laser scanning microscope. Images correspond to 4 layers of a z-stack and were processed using ImageJ software (1.48v; National Institutes of Health).

10. *In situ* hybridization

In situ hybridization was performed in paraffin embedded tissue sections and was executed as previously described (Bernal and Guadaño-Ferraz, 2002). Shortly, *Dio2* antisense and sense riboprobes were synthesized in the presence of ^{35}S-UTP from the D2 cDNA *Rattus norvegicus* template (NM_031720.4; spanning nucleotides 535–901) as described in Guadaño-Ferraz et al. (1997) which shares a 95% of similarity with *Mus musculus*. After deparaffination, hydration and permeabilisation, sections were incubated with $9x10^6$ cpm/500µl of the labelled antisense riboprobes and sense riboprobes as negative controls overnight at 55° C. Non-specific probe binding and the excess probe was washed away in buffers of decreasing salt concentrations at increasing temperatures up to 68° C. Sections were then dehydrated in ascending alcohols with NH_4OAc 0.3M and exposed to photographic film (BioMax, Kodak). After exposure for 60 days, the films were developed with Kodak D19 and fixed with fixative (Agfa, G354). Controls for the specificity of the hybridization included the use of the antisense probe which gave no signal.

11. Nissl staining

Sections were deparaffinised and hydrated as explained above and were incubated 15 min with 0.1% toluidine blue (Merk, 115930). Then they were dehydrated in ascending alcohols, cleared in xylenes, and covered with hydrophobic mounting medium Depex (Serva, 18243). Histological analyses were made with Nikon Eclipse 80i microscope and photomicrographs were acquired with a Nikon DS-Fi1 digital camera as mentioned in the immunohistochemical and immunofluorescence procedures section.

12. Statistical Analysis

Differences between two groups were compared by two-tailed unpaired Student's t-tests. Difference between means of three or more independent samples was done by one-way analysis of variance (ANOVA) and the Bonferroni's posthoc test. To examine the effect of the two factors, such as treatment and genotype, data were analysed by two-way ANOVA and the Bonferroni's posthoc test.

In all of the cases the GraphPad software (www.graphpad.com) was used. Significant differences were represented as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. All other comparisons for which the significance is not shown were not significant.

RESULTS

PART I: Contribution of maternal hormones and foetal D2 to thyroid hormone brain economy during perinatal development in mice

The first goal was to study the relative contribution of maternal T3 and T4 on foetal brain development. For this aim, euthyroid pregnant dams were treated with either 1.5 µg of T3/100 g BW or 8 µg of T4/100 g BW in the drinking water from E12 to the day prior to birth E18. In contrast to previous similar studies conducted in rat (Grijota-Martínez et al., 2011, Calvo et al., 1990), in the present study a state of global hypothyroidism was not induced. Antithyroid drugs Methymazole and $KClO_4$, commonly used to induce hypothyroidism, were not used in order to avoid the side effects not related to thyroid function and also to avoid blocking the foetal thyroid gland. Furthermore, maternal thyroidectomy was not performed as thyroidectomy in mice is not as common as in the rat, conceivably because of the difficulty of the microsurgical technique.

At E18 maternal plasma as well as foetal livers and cerebrums were collected to assess the effects of the treatments. The following groups were compared: Wt basal dams and foetuses (euthyroid dams); Wt + T3 dams and foetuses (euthyroid dams treated with T3) and Wt + T4 dams and foetuses (euthyroid dams treated with T4).

1.1 Peripheral effect of thyroid hormone treatment in dams and foetuses

The outcome of the treatment was checked by TH measurements in the dams and foetuses (**Figure 6**). The effects at the periphery were assessed by performing TH measurements in the dams' plasma and, considering the difficulties to obtain plasma samples from E18 foetuses, TH determinations were performed in foetal livers assuming that TH content in the liver reflects TH levels in plasma.

T3 treatment caused a 3-fold decrease in the maternal plasma T4 levels probably due to TSH suppression and this was reflected on the T4 foetal liver content which also decreased after treatment with T3 (**Figure 6 A, B**). In contrast, while T3 treatment increased T3 maternal plasma levels by 4-fold, it did not increase the T3 content in foetal liver (**Figure 6 D, E**).

T4 treatment greatly increased T4 levels in maternal plasma and this increase was reflected on foetal liver T4 content that also increased (**Figure 6 A, B**). After T4 treatment, T3

concentration increased in maternal plasma to similar levels to that attained by T3 treatment, however, T3 content did not increase in foetal liver (**Figure 6 D, E**).

The effects of T3 and T4 treatments on the expression of TH transporters on foetal liver were studied by gene expression analysis. The selected genes were two TH transporters (Lat1 and Lat2) that have been previously described to be up-regulated by T4 (Engels et al., 2015) and that are also expressed at E18 (Li et al., 2012). The expression of the TH transporter Mct8 that, although it is not upregulated by T4, is expressed in foetal liver (Engels et al., 2015, Li et al., 2012) was also monitored. The relative expression of these transporters at E18 versus their expression on later stages in life was also studied by comparing mRNA expression levels in the liver from basal Wt animals at E18 and basal Wt at P30. The results showed that *Mct8* expression in the liver was higher in P30 animals than in E18 embryos and its expression was not regulated after treatment at E18 (**Figure 7 A, B**). In contrast, expression of both *Lat1* and *Lat2* transporters at E18 was diminished after treatment with T3 and T4, although this decrease was only statistically significant when treating with T4 (**Figure 7 C, E**). This differs from what has been observed in 4-month-old mice where T4 treatment up-regulated the expression of these two transporters (Engels et al., 2015). However, consistent with previous studies (Li et al., 2012), the expression of *Lat1* and *Lat2* was highly enriched in E18 embryos in comparison to P30 mice (**Figure 7 D, F**).

Why T3 content in the foetal liver did not increase in accordance to the increase in the T4 content when treating the mothers with T4, might be explained by the lack of D1 activity in the foetal liver. In D1 activity assays, D1 activity was not detected in any of the experimental conditions (data not shown) showing that D1 is not present in foetal euthyroid liver and that is not inducible by hyperthyroid conditions.

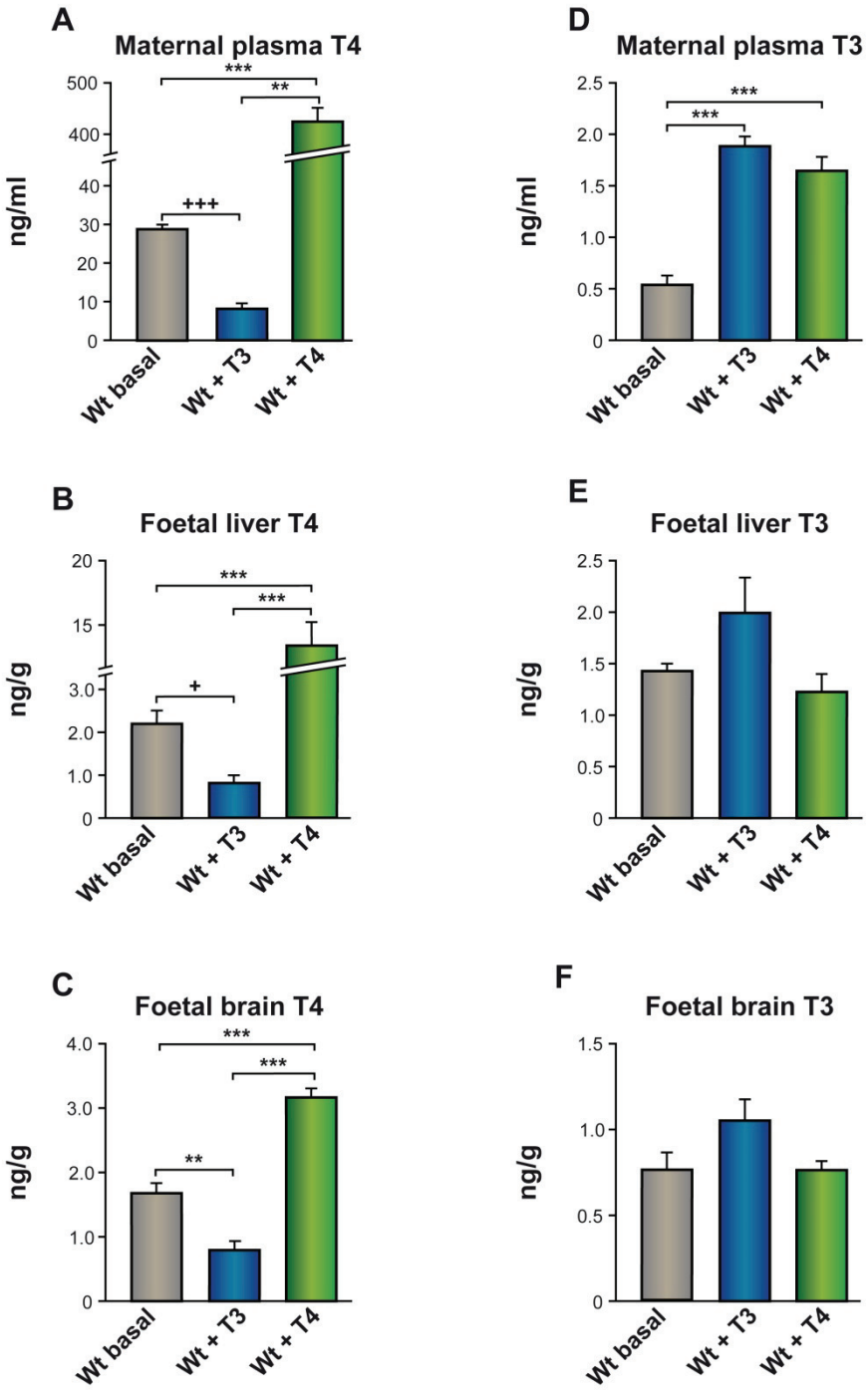


Figure 6. Thyroid hormones in dams and E18 foetuses. Euthyroid pregnant dams (Wt basal) treated with 1.5 µg T3/100 g BW/day (Wt + T3) or with 8 µg T4/100 g BW/day (Wt + T4) from E12 to E18. The left panels show changes in T4 levels in maternal plasma **(A)** and T4 content in foetal liver and brain **(B, C)** while right panels show T3 levels in maternal plasma **(D)** and T3 content in foetal liver and brain **(E, F)**. Data are expressed as means ± SE. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were determined by One-way ANOVA and Bonferroni's post hoc test. $^{+}p < 0.05$ and $^{+++}p < 0.001$ were determined by two-tailed unpaired Student's t-test.

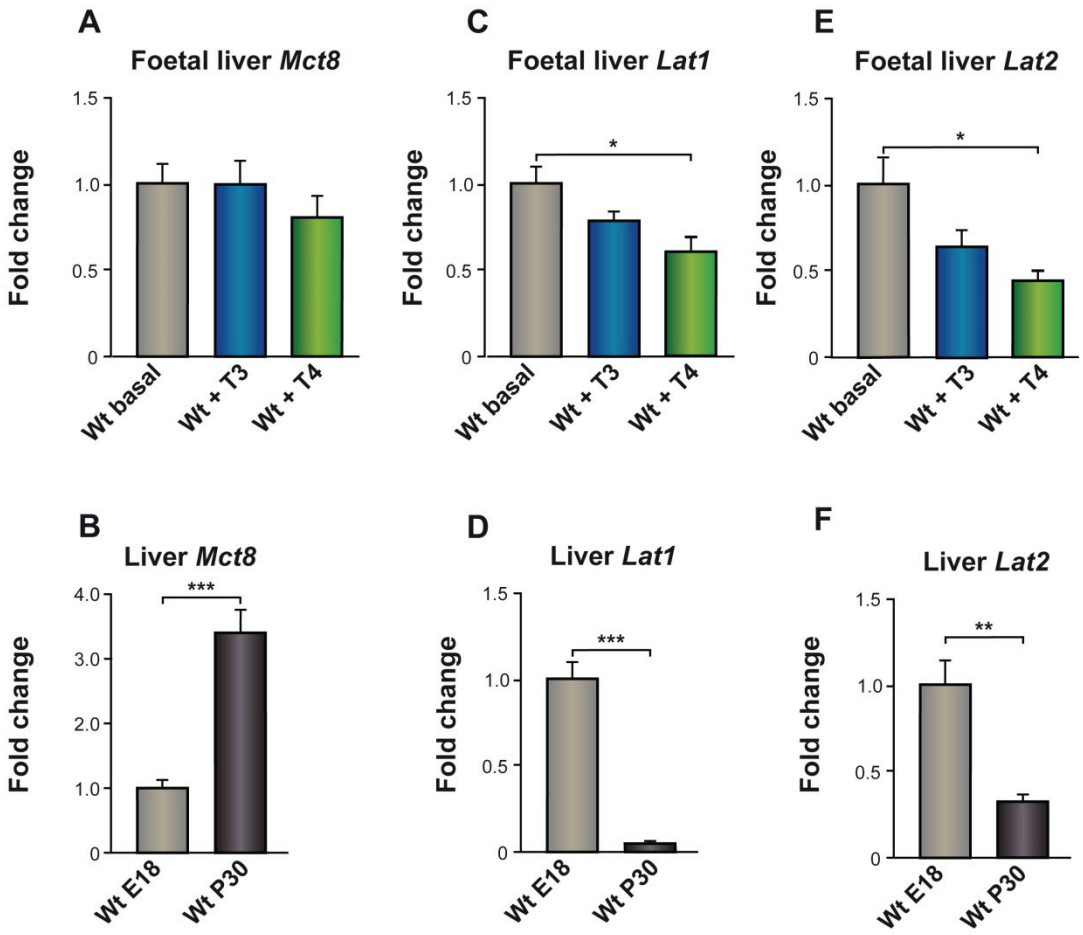
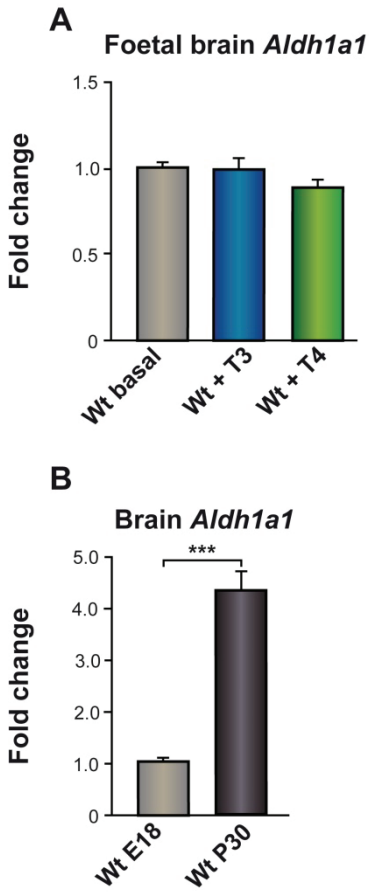


Figure 7. Gene expression analysis of thyroid hormone transporters in liver. Upper panels show comparison of the effect of T3 and T4 treatment on the expression levels of Mct8, Lat1 and Lat2 transporters in E18 foetal livers **(A, C, E)** while lower panels show the relative expression of these transporters at E18 vs P30 in liver **(B, D, F)**. Measurements were obtained by qPCR and the data are expressed relative to 18S RNA as means ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001 were determined by One-way ANOVA and Bonferroni's post hoc test for the upper panels and by two-tailed unpaired Student's t-test for the lower panels.

1.2 Effects of maternal thyroid hormone treatment in foetal brain

In the foetal brain, T4 content reflected the peripheral T4 status of the foetus: T3 treatment decreased the foetal liver T4 content by approximately 3-fold and by 2-fold the brain T4 content compared to the basal group and, on the other hand, T4 treatment induced a 6-fold increase in the foetal liver T4 content and a 1.75-fold increase in the brain T4 content compared to the basal group (**Figure 6 C**). Despite these variations in the T4 brain content, T3 content remained constant and close to normal control values (**Figure 6 F**).



The expression of the T3-resposive gene *Aldh1a1* (Hernandez et al., 2012) was consistent with the constant content of T3 in the brain regardless of the variations in the T4 content. *Aldh1a1* expression in the foetal brain was not affected in any of the experimental conditions (**Figure 8 A**). The comparison of basal *Aldh1a1* mRNA expression levels in Wt brain between E18 and P30 animals revealed that its expression is enriched at P30 (**Figure 8 B**).

As brain T3 levels are highly dependent on brain T4 content the results suggests the presence of important regulatory mechanisms that maintain brain T3 homeostasis. Two of these regulatory mechanisms in the brain could be the regulation of the TH transport to the brain and the regulation of deiodinases activities.

Figure 8. Gene expression analysis of the T3-dependent gene *Aldh1a1* in brain. Comparison of the effect of T3 and T4 treatment on the expression levels of *Aldh1a1* in E18 brains **(A)** and the relative expression of this gene at E18 vs P30 in brain **(B)**. Measurements were obtained by qPCR, and the data are expressed relative to 18S RNA as means ± SE. ***p < 0.001 were determined by One-way ANOVA and Bonferroni's post hoc test for figure A and by two-tailed unpaired Student's t-test for figure B.

1.2.1 Thyroid hormone transporters in foetal brain after maternal treatment

To evaluate the potential regulation of TH transporters as a compensatory mechanism that ensures appropriate delivery of TH to the brain under variations on the concentrations of T4, the expression of the main TH transporters in the brain was studied by qPCR. Furthermore, the relative expression of these transporters at E18 in contrast to later stages in life was also assessed by comparing mRNA expression levels from basal Wt animals at E18 and P30.

None of the studied transporters (Mct8, Oatp1c1 and Lat2) was differentially regulated after T3 or T4 treatment in comparison to the basal Wt (**Figure 9 A, C, E**), suggesting that differential expression of these transporters is not involved in regulating TH delivery to the brain at E18 after maternal treatment.

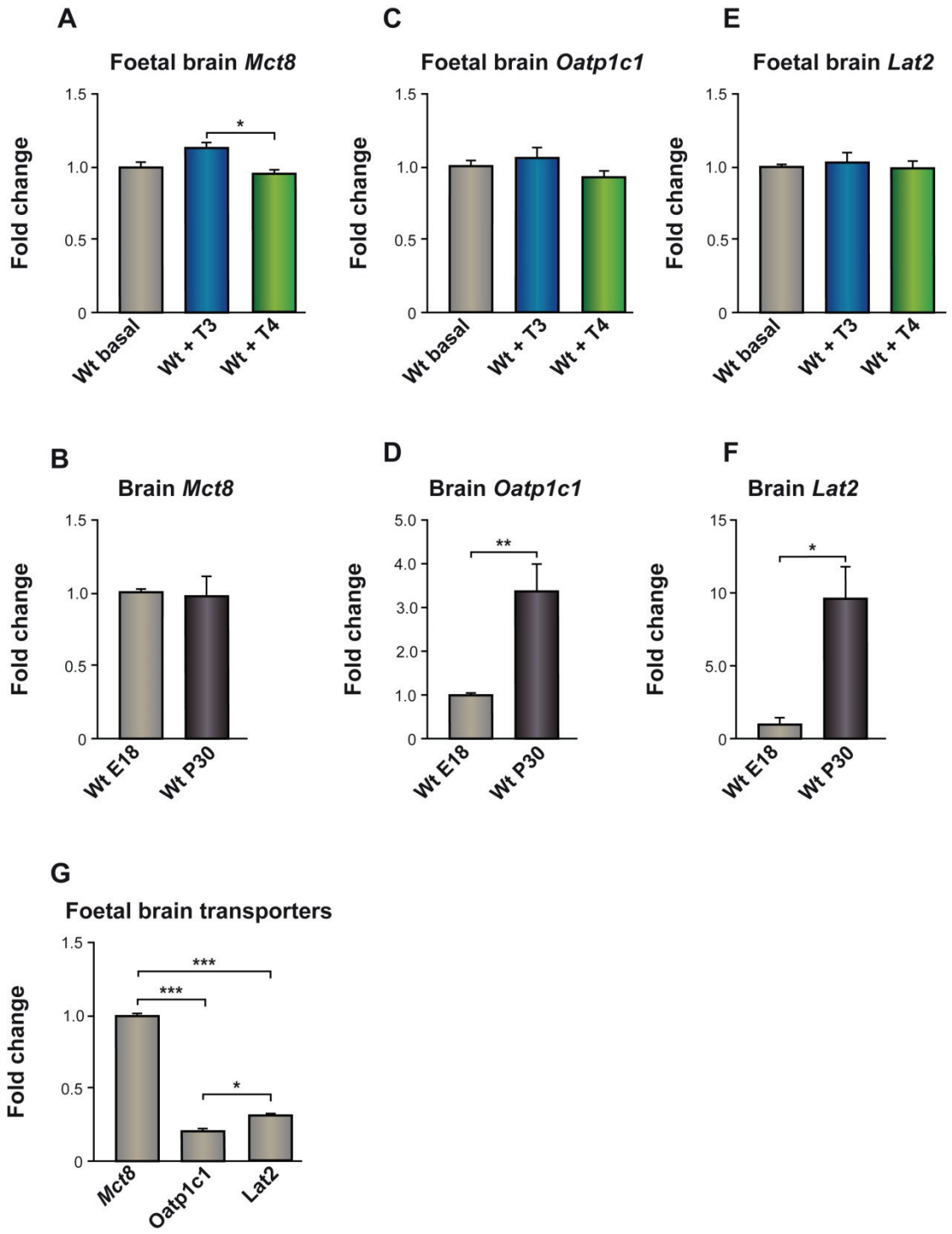


Figure 9. Gene expression analysis of thyroid hormone transporters in brain. Upper panels show comparison of the effect of T3 and T4 treatment on the expression levels of *Mct8*, *Oatp1c1* and *Lat2* transporters in E18 foetal brains **(A, C, E)** while lower panels show the relative expression of these transporters at E18 vs P30 in brain **(B, D, F)**. **(G)** Relative mRNA expression of the TH transporters Mct8, Oatp1c1 and Lat2 in foetal E18 brain. Measurements were obtained by qPCR, and the data are expressed relative to 18S RNA as means ± SE. *$p < 0.05$ and **$p < 0.01$ were determined by One-way ANOVA and Bonferroni's post hoc test for comparisons among 3 samples and by two-tailed unpaired Student's t-test for comparisons among 2 samples.

While *Mct8* mRNA expression was the same at E18 and at P30 (**Figure 9 B**), the expression of both *Oatp1c1* and *Lat2* was greatly increased in postnatal P30 animals in comparison to E18 embryos (**Figure 9 D, F**). Furthermore, the relative expression of these 3 transporters at E18 was also studied by qPCR. Mct8 appeared to be the most abundant transporter as *Mct8* expression was much higher than *Lat2* and *Oatp1c1* in foetal E18 brains (**Figure 9 G**).

1.2.2 Deiodinases in foetal brain

The possible contribution of deiodinases D3 and D2 activities to the maintenance of T3 brain content was also assessed.

D3 plays an essential role in the control of T3 concentration in developing tissues (Bates, 1999; Kester, 2004). As there is a good correlation between D3 activity and *Dio3* mRNA content of tissues (Gereben et al., 2008), *Dio3* mRNA quantification by qPCR was used to analyse the role of D3. This analysis showed that *Dio3* expression in the foetal brain did not change after treating the mothers with T3 or T4 (**Figure 10 A**). This indicates that T3 foetal brain homeostasis is not due to variations in the degradation of T3 by D3. Furthermore, the relative Wt basal expression of *Dio3* at E18 was not different from its expression at further stages as P30 (**Figure 10 B**).

As D2 activity is highly regulated by T4, activity assays were performed in foetal brain to determine D2 activity under the different conditions. T3 treatment to the mothers resulted in a reduction of foetal brain T4 content and D2 activity was accordingly increased. On the other hand T4 treatment to the mothers led to a pronounced increase in the T4 foetal brain content and, under this condition, D2 activity was markedly inhibited (**Figure 10 E**). This indicates that D2 activity is present in mouse foetal brain and is highly responsive to brain T4 concentrations.

Because D2 can also be regulated at the genomic level by T3 (Burmeister et al., 1997, Croteau et al., 1996, Guadaño-Ferraz et al., 1999), *Dio*2 expression was studied by qPCR. *Dio*2 gene expression did not change in response to T3 or T4 treatments (**Figure 10 C**) indicating that, under the present conditions, regulation of D2 activity at E18 is not at the transcriptional level. Moreover, despite the key role that D2 plays at E18 its expression was much more abundant in later stages such as P30 (**Figure 10 D**).

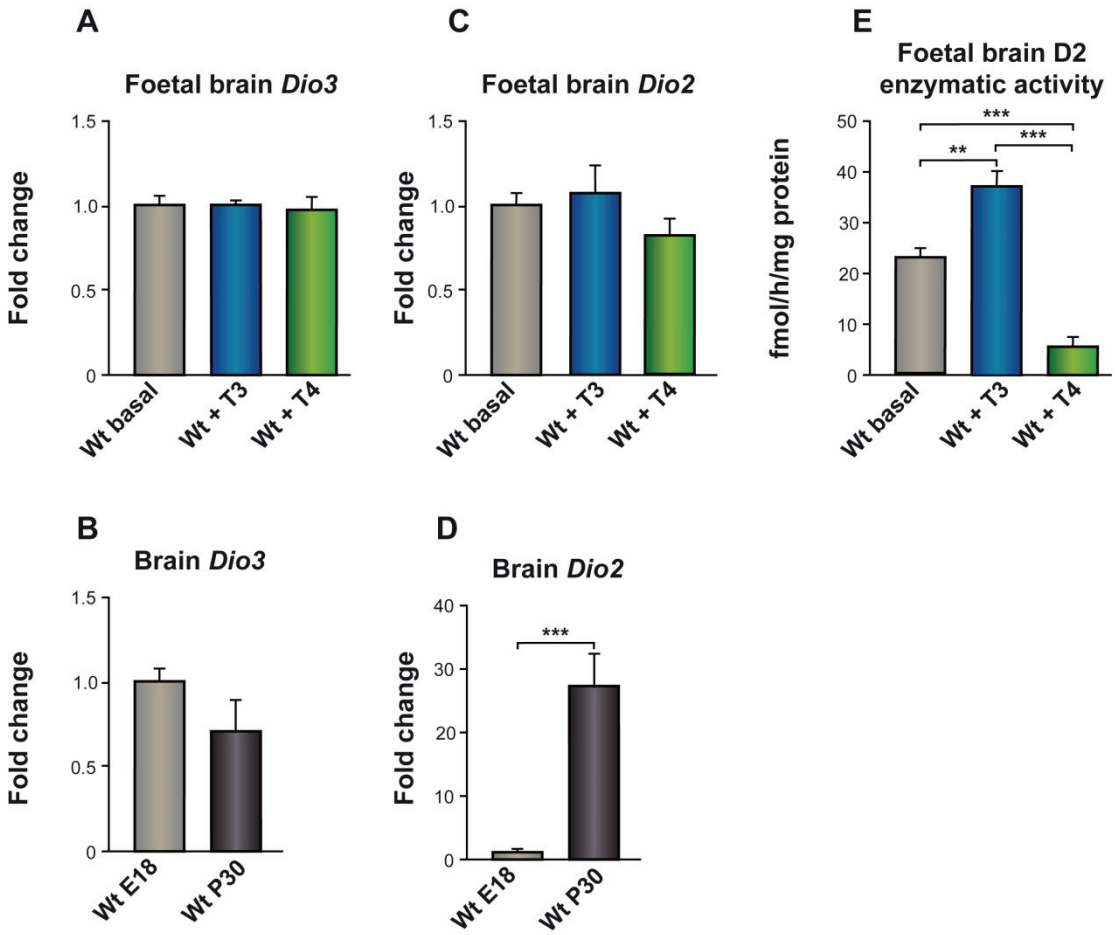


Figure 10. **Deiodinases 3 and 2 in brain**. Figures **A** and **C** compare the effect of T3 and T4 treatment on the expression levels of *Dio3*, and *Dio2* in E18 foetal brains. Figures **B** and **D** show the relative expression of these deiodinases at E18 vs P30 in brain. Measurements were obtained by qPCR, and the data are expressed relative to 18S RNA. Figure **E** shows the effect of T3 and T4 treatment on D2 enzymatic activity in foetal E18 brains as means ± SE. **p < 0.01 and ***p < 0.001 were determined by One-way ANOVA and Bonferroni's post hoc test for figures A, C and E and by two-tailed unpaired Student's t-test for B and D.

Due to the key role of D2 in the regulation of T3 brain levels, it was of great importance to analyse its expression at a histological level. Despite the efforts that were put into validating anti-D2 antibodies (Santa Cruz, sc-98716 and sc-69559) to study D2 protein expression in mouse brain through western blot and immunohistological procedures, the specificity of these antibodies for D2 could not be satisfactorily proven. Instead, *Dio2* mRNA expression was analysed by *in situ* hybridization both at foetal stages (E15 and E18) and at early postnatal stages (P1 and P3) to characterise *Dio*2 expression pattern throughout development. Film autoradiograms from representative coronal sections at different brain levels are shown in **Figure 11** along with the corresponding histological sections stained with Nissl.

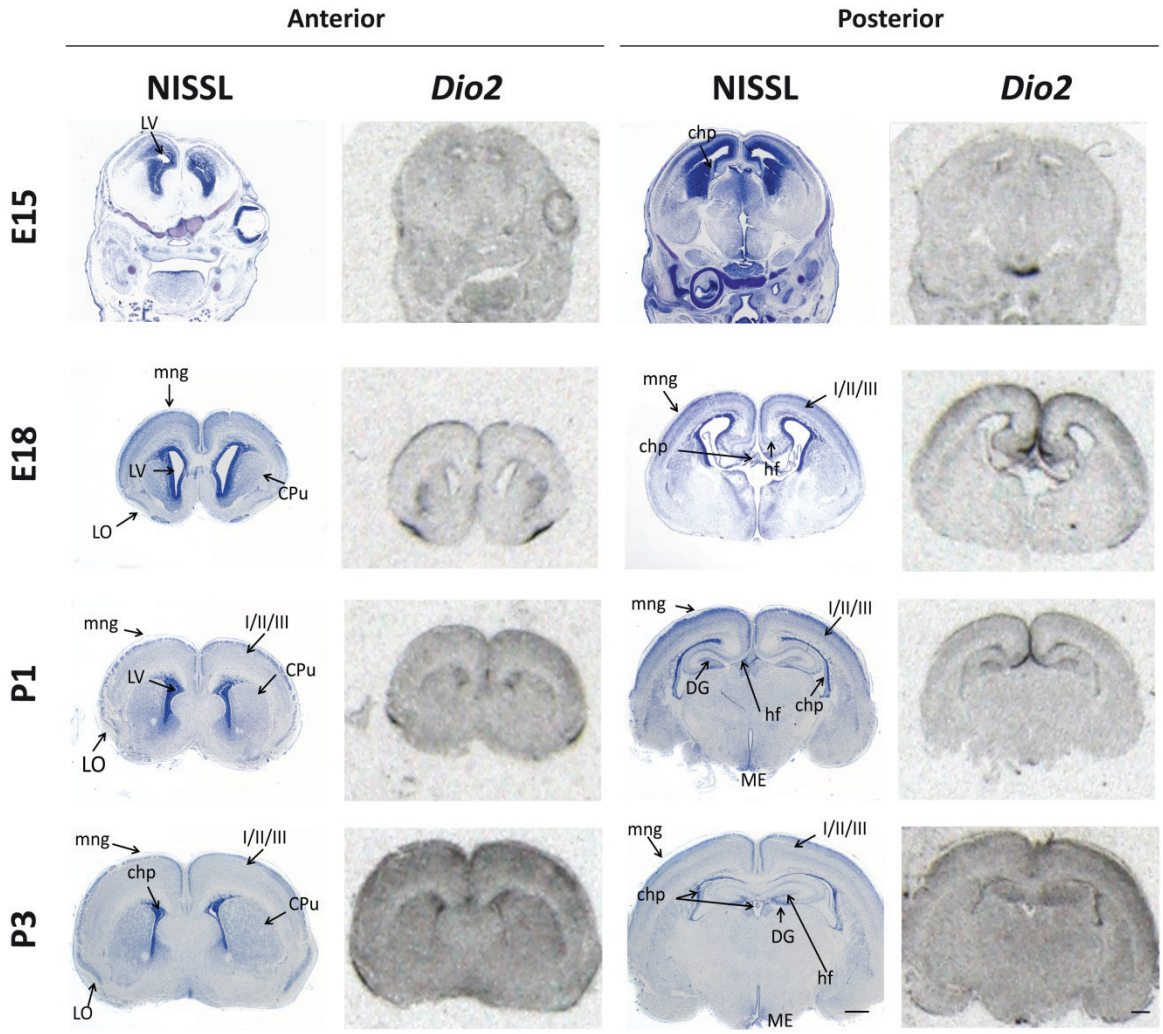


Figure 11. ***In situ*** **hybridization on sections of E15, E18, P1 and P3 perinatal mouse brain showing the regional distribution of *Dio2* mRNA**. Representative coronal sections of anterior brain levels are shown on the left and representative coronal sections of posterior brain levels are shown on the right. Film autoradiograms along with the corresponding histological sections stained with Nissl are shown in each case. I, II, III, superior layers of the cerebral cortex; chp, choroid plexus; CPu, caudate putamen (striatum); DG, dentate gyrus; hf, hippocampal fissure; LO, lateral olfactory tract; LV, lateral ventricles; ME, median eminence and mng, meninges. Scale bars 500 µm.

At E15 *Dio2* mRNA expression was visible in the ependymal layer of the lateral ventricles (LV) and the choroid plexus (chp). Later on it was also detected in the meninges (mng), the striatum (CPu), the lateral olfactory tract (LO) and the upper layers of the cerebral cortex (I, II, III). At P1 and P3, *Dio2* expression was also detected in the hippocampus, especially the dentate gyrus (DG), the hippocampal fissure (hf) and the median eminence (ME) resembling the already described *Dio2* expression in rat at P15 (Guadaño-Ferraz et al., 1997). *Dio2* expression in the ependymal layer of the lateral ventricles, the choroid plexus and the

meninges suggests that D2 might be mediating the conversion of T4 into T3 in the brain barriers from as early as E15 providing an essential source of T3 to the brain during mouse foetal development.

1.3 Role of D2 in Mct8 deficiency during early brain development

Given the apparent role of D2 in the local generation of brain T3 during development, we analysed its possible contribution to the brain hyperthyroidism that paradoxically present *Mct8*KO mice during the perinatal period. To this aim, the expression of a set of T3-dependent genes was compared in the genotypes Wt, *Mct8*KO, *Dio2*KO and the double KO *Mct8/Dio2*KO animals.

*Mct8*KO mice display a brain hyperthyroid state from at least E18 (Ferrara et al., 2013) that is more obvious at early postnatal stages (Nuñez et al., 2014). We compared the expression of the T3-responsive genes *Hr*, *Shh* and *Klf9* in the cerebral cortex of Wt, *Mct8*KO, *Dio2*KO and *Mct8/Dio2*KO animals on P3. This was done by comparing animals from the same litter so the following groups were prepared: Wt and *Mct8*KO; Wt and *Dio2*KO and finally *Dio2*KO and *Mct8/Dio2*KO.

On P3, *Mct8*KO animals displayed a state of brain hyperthyroidism with elevated expression levels of *Hr* and *Shh* in comparison to Wt animals. *Klf9* only showed a modest non statistically significant increased expression. On the contrary *Dio2*KO mice presented a state of brain hypothyroidism as the expression levels of *Hr* and *Shh* were decreased in comparison to Wt animals. In this situation, *Klf9* expression was slightly decreased, although this decrease was not statistically significant. The expression profiles of these T3-dependent genes in the double *Mct8/Dio2*KO were the same as in the single *Dio2*KO animals; hence *Mct8/Dio2*KO presented a state of brain hypothyroidism in comparison to Wt animals. Expression of the gene encoding for the TH transporter Mct8 did not change in the absence of D2 (**Figure 12**).

Juvenile and adult *Mct8*KO mice have an increased D2 activity in brain as a consequence of the decreased T4 supply (Dumitrescu et al., 2006, Trajkovic et al., 2007). Whether an increased D2 activity in the P3 *Mct8*KO mice could be responsible for an increased T3 formation and local brain hyperthyroidism was assessed. However, D2 enzymatic activity assays revealed that D2 activity was not altered in the cerebral cortex of *Mct8*KO animals at P3 in comparison to Wt littermates. D2 activity in *Mct8*KO mice at P30 was remarkably increased in comparison to the Wt as already described (**Figure 13**).

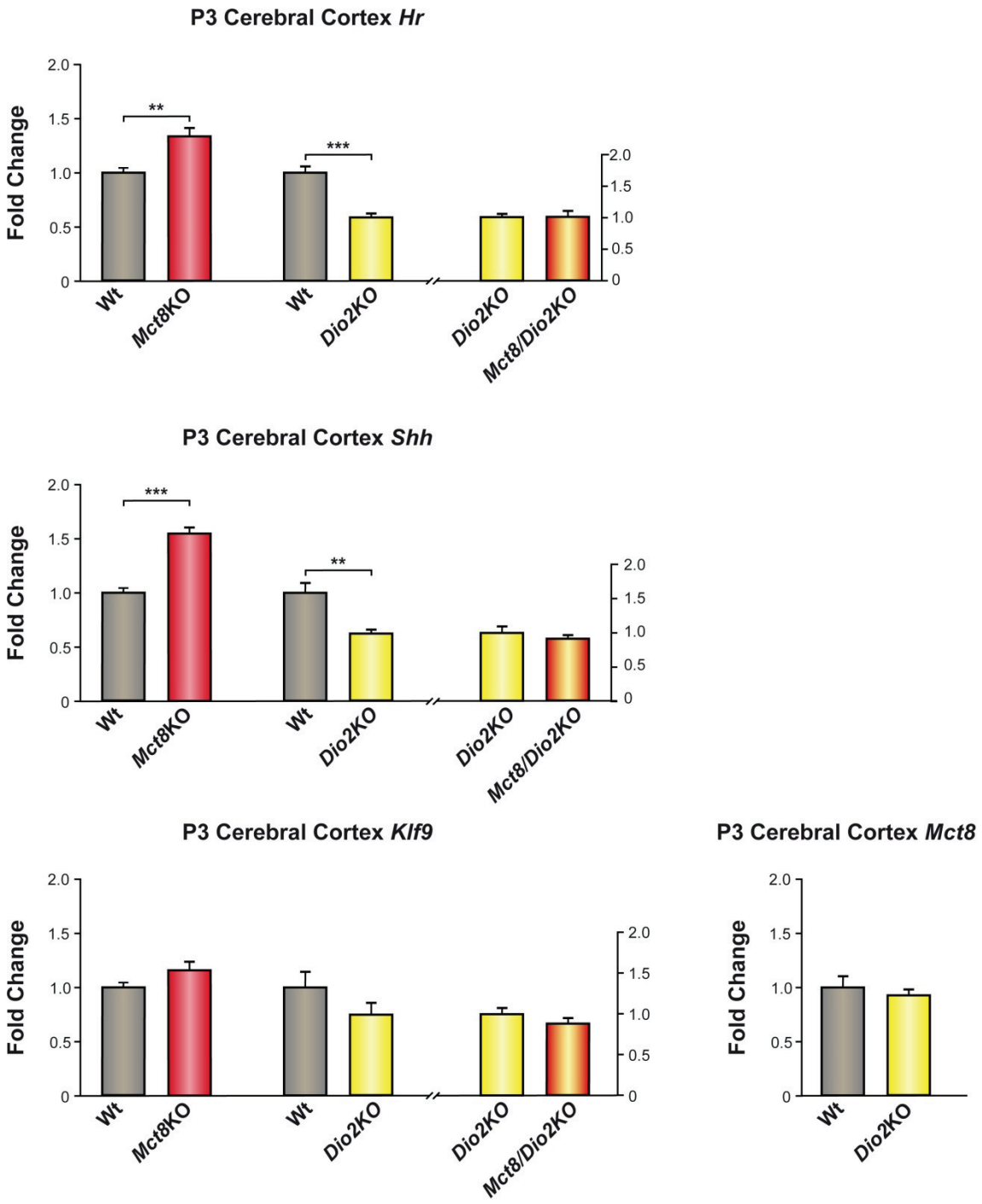


Figure 12. Gene expression analysis of T3-dependent genes (*Hr*, *Shh and Klf9*) in the cerebral cortex of Wt, *Mct8*KO, *D2*KO and double *Mct8/D2*KO mice at P3. Animals from the same litter were compared: Wt and *Mct8*KO; Wt and *D2*KO; *D2*KO and *Mct8/D2*KO. The bottom of the panel shows gene expression analysis of TH transporter Mct8 between Wt and *D2*KO animals in the cerebral cortex at P3. Measurements were obtained by qPCR, and the data are expressed relative to 18S RNA as means ± SE. **p < 0.01 and ***p < 0.001 were determined by two-tailed unpaired Student's t-test using the respective controls for each case.

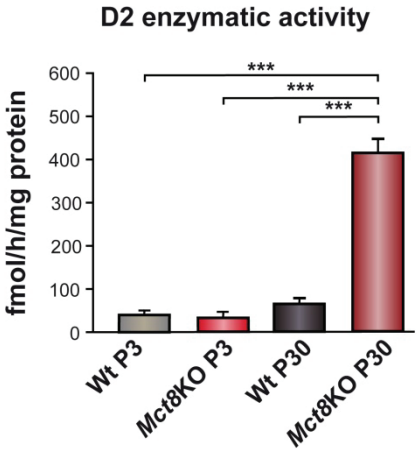


Figure 13. Enzymatic D2 activity in cerebral cortices of Wt and *Mct8*KO animals at P3 and at P30. Data are expressed as means ± SE. ***p < 0.001 was determined by One-way ANOVA and Bonferroni's post hoc test.

PART II: Therapeutic approaches for MCT8 deficiency

As introduced, the current therapeutic strategies used in MCT8 deficiency have been successful in normalising the circulating levels of T3 which alleviates the thyrotoxicosis of peripheral tissues; however, none of the treatments has improved the neurological deficits in these patients.

In this part of the thesis we have used *Mct8*KO mice to perform preclinical studies to test different approaches in order to develop therapeutic strategies for MCT8 deficiency. Despite the fact that *Mct8*KO mice do not reproduce the neurological deficits of the patients they provide a good model to assess the effects of different therapies. *Mct8*KO mice replicate the patient's endocrine syndrome, which enables the evaluation of the effects of treatments on serum TH levels. Furthermore, even though *Mct8*KO animals do not faithfully replicate the severe neurological impairments of patients, T3 and T4 content in the brain is decreased approximately by half. This leads to alterations in the expression of some T3-dependent genes (Morte et al., 2010) that can be used as markers to evaluate therapeutic actions in the brain. Moreover, *Mct8*KO mice have already been successfully used to study the effect of TH

analogues as possible treatments for MCT8 deficiency (Di Cosmo et al., 2009, Ferrara et al., 2014, Ferrara et al., 2015).

Because MCT8 deficient patients present cerebral hypothyroidism in combination with peripheral hyperthyroidism the design of an appropriate treatment is rather challenging. Therapeutic strategies should improve the neurological defects derived from TH deficiency in the brain and improve the peripheral thyrotoxicosis. As the main restriction for T3 entry into the target neural cells is at the BBB the approaches used here have focused on overcoming the limitation at the BBB by the use of TH analogues, of gene therapy strategies and by using novel administration routes for brain TH replacement therapy.

2.1 Thyroid hormone analogues: TRIAC

2.1.1 Background

TRIAC is a natural TH metabolite that displays properties that make it a good candidate as a therapeutic agent in MCT8 deficiency. To start with, TRIAC binds with the same affinity as T3 to the TRα and with higher affinity to the TRβ (Messier and Langlois, 2000). Furthermore, TRIAC is already available for clinical use and there is extensive clinical experience, as it has successfully been used in patients with thyroid cancer (Müeller-Gäertner and Schneider, 1988) and mutations in *THRB* (Anzai et al., 2012, Stagi et al., 2014). Moreover, *in vitro* and *in vivo* studies in mice have demonstrated that (i) TRIAC is generated from TETRAC by D2 activity; (ii) that it is metabolized by D3 in the same way as T3; but most importantly that (iii) it is transported into brain cells by other transporters than MCT8, (iv) that induces similar neuronal gene responses as T3 and (v) that is able to restore neuronal differentiation in the hypothyroid brain (Horn et al., 2013, Kersseboom et al., 2015).

However, the fact that TRIAC produces similar gene responses as T3 was assessed only *in vitro*, and the *in vivo* studies concluding that TRIAC restores neuronal differentiation in the hypothyroid brain of the athyroid Pax8 KO and the double *Mct8*/*Oatp1c1* KO mice were obtained using high doses of TRIAC (200 – 400 ng/g BW), while lower doses of 50 ng/g BW only had modest effects (Kersseboom et al., 2015). As in human clinical trials TRIAC has been used at doses ranging from 5 to 50 µg/kg BW/day (Müeller-Gäertner and Schneider, 1988, Anzai et al., 2012, Stagi et al., 2014), here the effect of therapeutic doses of TRIAC (30 ng/g BW/day) on *Mct8*KO mice was evaluated to characterise its effects as a potential therapeutic

agent in MCT8 deficient patients. And as most patients are diagnosed after birth, the study was performed in juvenile mice in order to have comparable conditions to patients.

For these reasons the design of the treatment consisted on the administration of therapeutic doses of 30 ng of TRIAC/g BW/day in the drinking water from P21 until P30 to Wt and *Mct8*KO mice. At P30 brains and peripheral tissues were harvested after saline perfusion to assess TRIAC effect so the following groups were compared: Basal Wt; Wt + TRIAC; Basal *Mct8*KO and *Mct8*KO + TRIAC.

2.1.2 Effects of TRIAC on plasma thyroid hormone levels

Because patients suffer from peripheral tissue hyperthyroidism, the initial aim was to assess the effects of TRIAC on plasma TH levels.

TRIAC treatment increased nearly by 3-fold TRIAC plasma levels in comparison to untreated control animals, and reached the same concentration in both the Wt- and *Mct8*KO-treated mice (**Figure 14 A**). Plasma T4 levels in the untreated *Mct8*KO animals were decreased to nearly half the levels of the Wt, as already characterised in Mct8 deficiency (Dumitrescu et al., 2006, Trajkovic et al., 2007). TRIAC treatment drastically reduced the plasma T4 levels more than 70% in the treated animals of both genotypes in comparison to their respective basal controls (**Figure 14 B**), so T4 levels in TRIAC-treated *Mct8*KO animals were 6 times lower than in untreated Wt animals. TRIAC is known to have TSH-inhibitory effects (Beck-Peccoz et al., 1988, Bracco et al., 1993, Everts et al., 1994) causing a decrease of plasma T4 (Medina-Gomez et al., 2008). Although TSH was not measured in this study, the decrease in the T4 plasma levels both in the Wt and the *Mct8*KO animals suggests that Mct8 deficiency does not interfere with the feedback regulation of TSH by TRIAC. Basal plasma T3 levels were significantly higher in *Mct8*KO compared with Wt animals as previously described. Remarkably, TRIAC treatment decreased T3 in the *Mct8*KO to normal levels while it did not affect T3 levels in the Wt mice (**Figure 14 C**).

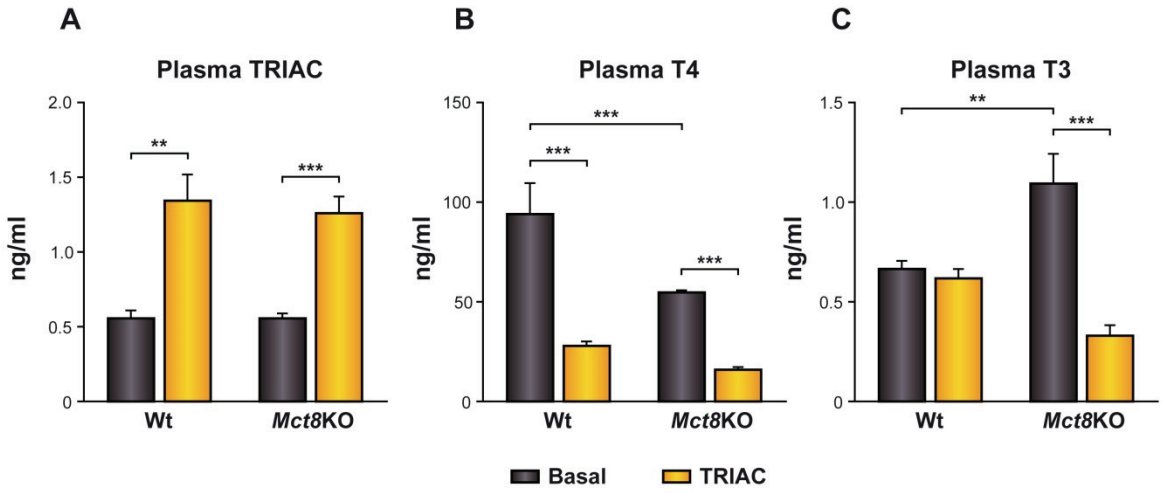


Figure 14. **Triiodothyroacetic acid (TRIAC, A), T4 (B) and T3 (C) plasma levels** in Wt and *Mct8*KO mice in basal conditions and after treatment with 30 ng/g BW/day of TRIAC. Data are expressed as means ± SE. **p < 0.01, and ***p < 0.001 were determined by two-way ANOVA and Bonferroni's post hoc test, the two factors being genotype and treatment.

2.1.3 Effects of TRIAC on the liver and heart

To further analyse the effects of TRIAC on peripheral organs we studied D1 activity and *Dio1* mRNA expression in the liver as D1 has been shown to be a sensitive marker of the peripheral thyroid status in the mouse (Zavacki et al., 2005). The study of D1 enzymatic activity in the liver displayed the well-characterised increase of this enzyme in *Mct8*KO animals compared with the Wt animals (Dumitrescu et al., 2006, Trajkovic et al., 2007). TRIAC treatment increased D1 activity both in the Wt and *Mct8*KO animals (**Figure 15 A**) despite a decrease in the plasma T3 levels, probably due to the effect of TRIAC as a TR agonist. Consistent with the D1 activity results, *Dio1* mRNA expression levels were increased in the *Mct8*KO compared with the Wt animals. TRIAC treatment increased *Dio1* expression 5-fold in the Wt mice and 1.5-fold in the *Mct8*KO mice in comparison to their basal levels (**Figure 15 B**), confirming the thyromimetic activity in the liver. Taken together, the findings indicate that TRIAC, similar to T3, has a direct effect on the liver regulating *Dio1* mRNA expression and D1 activity.

TRIAC effect in the heart was evaluated by analysing the expression of the T3-responsive gene *Serca2a*. TRIAC treatment did not have an effect on the expression of *Serca2a* neither in the Wt or the *Mct8*KO animals (**Figure 15 C**).

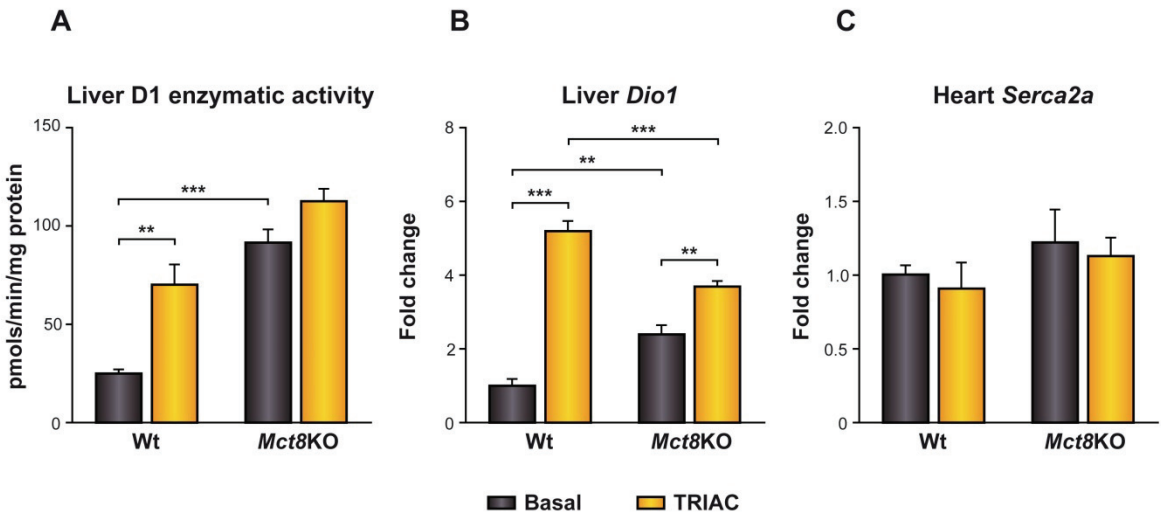


Figure 15. Liver D1 enzymatic activity (A) and gene expression analysis of *Dio1* (B) in the liver and *Serca2a* (C) in the heart in Wt and *Mct8*KO mice in basal conditions and after TRIAC treatment. Gene expression measurements were obtained by qPCR, and the data are expressed relative to 18S RNA as means ± SE. **p < 0.01, and ***p < 0.001 were determined by two-way ANOVA and Bonferroni's post hoc test, the two factors being genotype and treatment.

2.1.4 Effects of TRIAC in the brain

As all the current available treatments for MCT8 deficiency have failed to improve the neurological condition, the ultimate goal was to analyse the effect of therapeutic doses of TRIAC on the CNS of *Mct8*KO animals to evaluate its potential as a therapeutic agent. To explore if the therapeutic doses of TRIAC used in this study reach the brain and are able to exert any function at the genomic level, the expression of T3-dependent genes was studied in two different regions of the brain: the cerebral cortex and the striatum. The genes selected for this study were the T3-responsive genes (Hernandez et al., 2012) *Hr* and *Cbr2* that are altered in Mct8 deficiency (Morte et al., 2010) as well as other genes that, although they are not altered in Mct8-deficient mice, they are T3-responsive genes: *Itih*3, *Aldh1a1*, and *Flywch*2 (Hernandez et al., 2012).

In the cerebral cortex (**Figure 16**, left panels) the expression of *Hr* and *Cbr*2 was decreased in the basal *Mct8*KO animals compared with the Wt controls, while the expression levels of *Itih*3, *Aldh1a*1, and *Flywch*2 did not vary between the basal Wt and the *Mct8*KO, consistent with previous studies (Morte et al., 2010). TRIAC treatment did not increase the expression levels of *Hr* or *Cbr*2 in the Wt or the *Mct8*KO animals, suggesting that the treatment is not able to

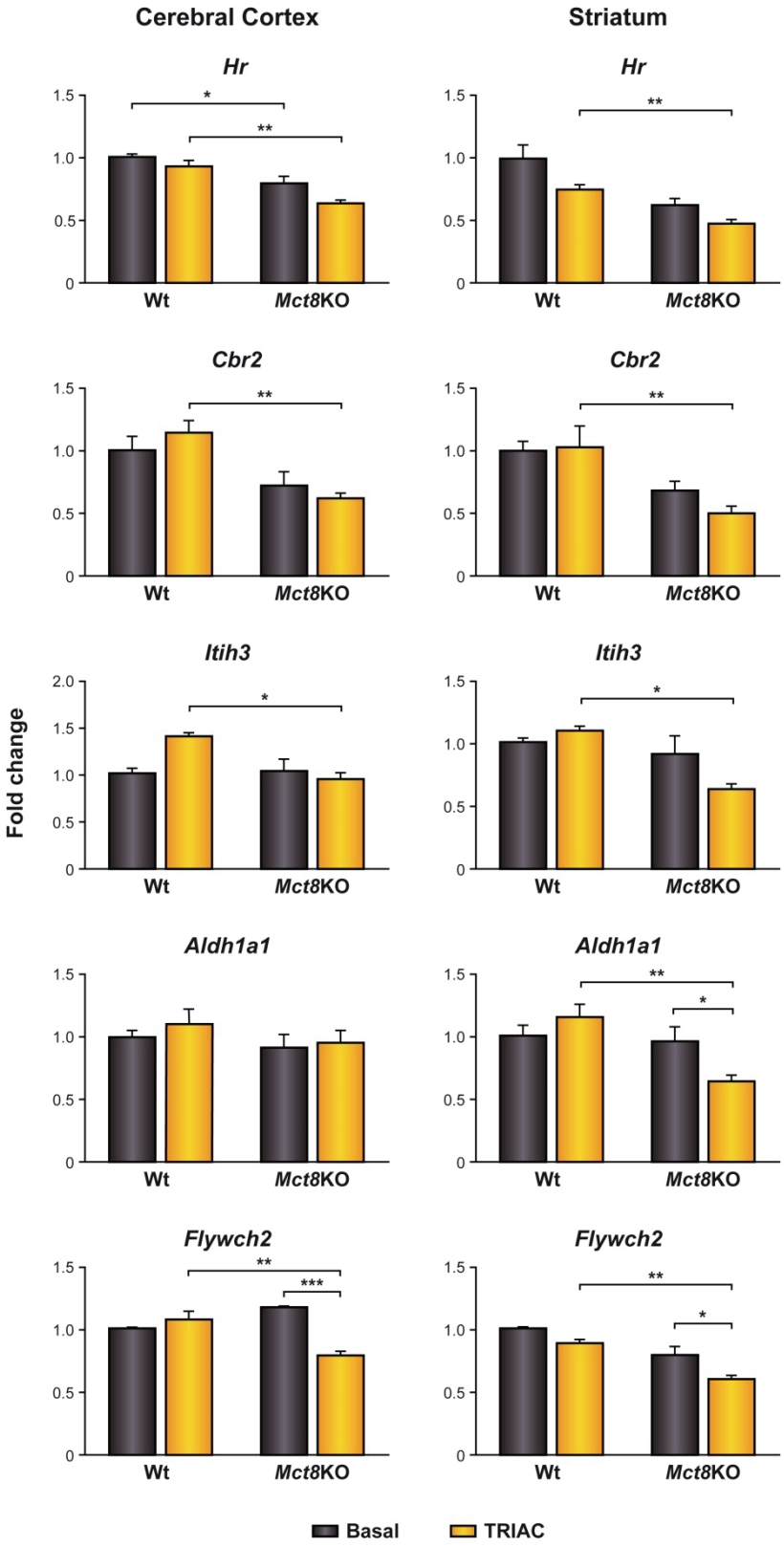


Figure 16. Gene expression analysis of T3-dependent genes in the brain: cerebral cortex (left panel) and the striatum (right panel) in Wt and *Mct8*KO mice in basal conditions and after TRIAC treatment. Measurements were obtained by qPCR, and the data are expressed relative to 18S RNA as means ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001 were determined by two-way ANOVA and Bonferroni's post hoc test, the two factors being genotype and treatment.

correct the gene expression defects of the *Mct8*KO mice. Likewise, TRIAC treatment did not increase the expression levels of *Itih*3 and *Aldh1a*1 in either the Wt or the *Mct8*KO animals compared with their basal controls. *Flywch*2 expression did not change after treatment in the Wt animals, however, contrary to expectations, TRIAC treatment decreased *Flywch*2 expression in the *Mct8*KO animals compared with untreated controls.

Outstandingly, in the striatum (**Figure 16**, right panels) the expression levels of all the selected T3-dependent genes were decreased in the TRIAC-treated *Mct8*KO animals compared with their basal controls, although the decrease was only statistically significant for *Aldh1a*1 and *Flywch*2.

Subsequently, the ability of TRIAC to reach the brain was evaluated by directly measuring TRIAC content in the cerebral cortex and the striatum of Wt and *Mct8*KO animals. In the cerebral cortex, TRIAC content was not increased after treatment either in Wt or *Mct8*KO mice; the small increase in TRIAC content was not statistically significant (**Figure 17 A**). The same was observed in the striatum (**Figure 17 B**) where TRIAC treatment did not increase TRIAC content in either genotype.

The CNS thyroidal status was further evaluated by determining the T3 content in the cerebral cortex and the striatum. In the cerebral cortex (**Figure 17 C**), the basal T3 content was decreased in *Mct8*KO in comparison to Wt mice, as already described (Dumitrescu et al., 2006, Trajkovic et al., 2007). After TRIAC treatment, T3 content decreased in both Wt and *Mct8*KO animals. The same was observed in the striatum (**Figure 17 D**), although in the *Mct8*KO mice the reduction in the T3 content after treatment was not statistically significant.

D2 enzymatic activity assays in the cerebral cortex (**Figure 17 E**) displayed the already characterised increase in D2 activity of *Mct8*KO animals compared with the Wt mice (Dumitrescu et al., 2006, Trajkovic et al., 2007) as a compensatory mechanism (Morte et al., 2010). However, D2 activity in *Mct8*KO animals did not change after treatment, probably due to saturation of its activity. In the Wt animals there was a small increase in D2 activity in the cerebral cortex after treatment, although this was not statistically significant.

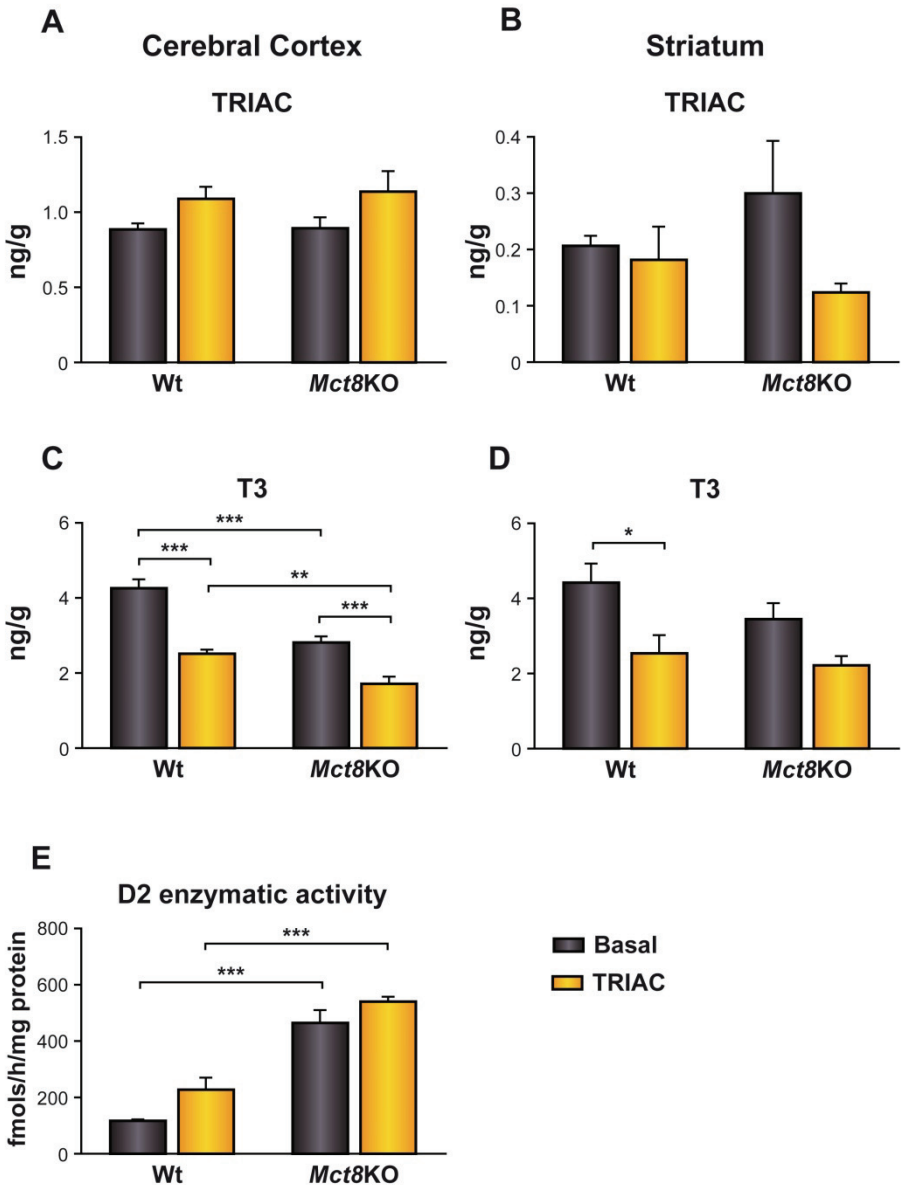


Figure 17. **TRIAC and T3 brain content**. TRIAC **(A)** and T3 **(C)** content in the cerebral cortex and TRIAC **(B)** and T3 **(D)** content in the striatum in Wt and *Mct8*KO mice in basal conditions and after TRIAC treatment. D2 enzymatic activity in the cerebral cortex of Wt and *Mct8*KO mice in basal conditions and after TRIAC treatment **(E)**. Data are expressed as means ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001 were determined by two-way ANOVA and Bonferroni's post hoc test, the two factors being genotype and treatment.

2.2 Gene therapy: Adeno-associated virus

2.2.1 Background

Gene therapy approaches by the use of adeno-associated virus (AAVs) were performed in collaboration with Dr. Samuel Refetoff's group in the University of Chicago. The findings

described in this background section were performed at the University of Chicago and only those relevant to the development of this thesis are described below. For complete details see Iwayama et al. (2016).

AAVs are small non-pathogenic virus that can infect various species (Schultz and Chamberlain, 2008). In the last years, its use in gene therapy has revealed several advantages such as a limited immune response, the ability to infect various cell types including post-mitotic cells, and persistence without integration into host chromosomes. Adeno-associated virus 9 (AAV9) transduces the brain with high efficiency after its delivery into both the blood stream and the CSF (Foust et al., 2009, Meyer et al., 2015). Therefore, the goal was to analyse if the transfer of human MCT8 (hMCT8) cDNA using AAV9 could correct the brain defects of *Mct8*KO mice.

AAV9 vectors associated with the short human MCT8 (ShMCT8) protein isoform or an empty vector (EV) were injected intravenously (IV; $4x10^{11}$ viral particles dose) or intracerebroventricularly (ICV; $3x10^{10}$ viral particles dose) to P1 *Mct8*KO and Wt animals. At P25, T3 (5 µg T3 /100 g BW) was administered via intraperitoneal injection daily for 4 consecutive days. At P28, brains were harvested after perfusion to study the effects of the delivery of ShMCT8. The following groups were compared: Wt + IV EV injection; *Mct8*KO + IV EV injection; *Mct8*KO + IV AAV9-ShMCT8 injection and *Mct8*KO + ICV AAV9-ShMCT8 injection.

Whether the ShMCT8 could be successfully delivered to the brain using either IV or ICV administration was assessed by western blotting. hMCT8 protein was not detected in EV injected animals. The ShMCT8 isoform showed similar amounts of monomers (60 kDa) and dimers (120 kDa), however, ICV injection of AAV9-ShMCT8 produced substantially more translated protein than IV injection of AAV9-ShMCT8 (**Figure 18 A**). Therefore, ICV delivery provided more hMCT8 protein than IV delivery.

Next, the functionality of hMCT8 delivery was assessed by determining T3 content in brain after 4 days of T3 administration as well as its effects on the expression of the T3-responsive gene, *Hr*. In *Mct8*KO mice, IV injection of AAV9-ShMCT8 increased brain T3 content 2-fold above the background of EV-injected *Mct8*KO mice while ICV injections of AAV9-ShMCT8 at did not increase the brain T3 content above background (**Figure 18 B**). This indicates that AAV9-ShMCT8 given IV, but not given ICV, can increase T3 content in cerebral tissue. In accordance to this, *Hr* mRNA expression measured by qPCR revealed that its expression increased 2-fold in comparison to the control mice (*Mct8*KO injected with EV) when the

AAV9-ShMCT8 was administered IV, but not ICV (Figure 18 C). Again, AAV9-ShMCT8 given IV, but not ICV, can increase the expression of the T3-responsive gene *Hr*. However, neither the increase in the T3 content or in the expression of *Hr* was similar to Wt values.

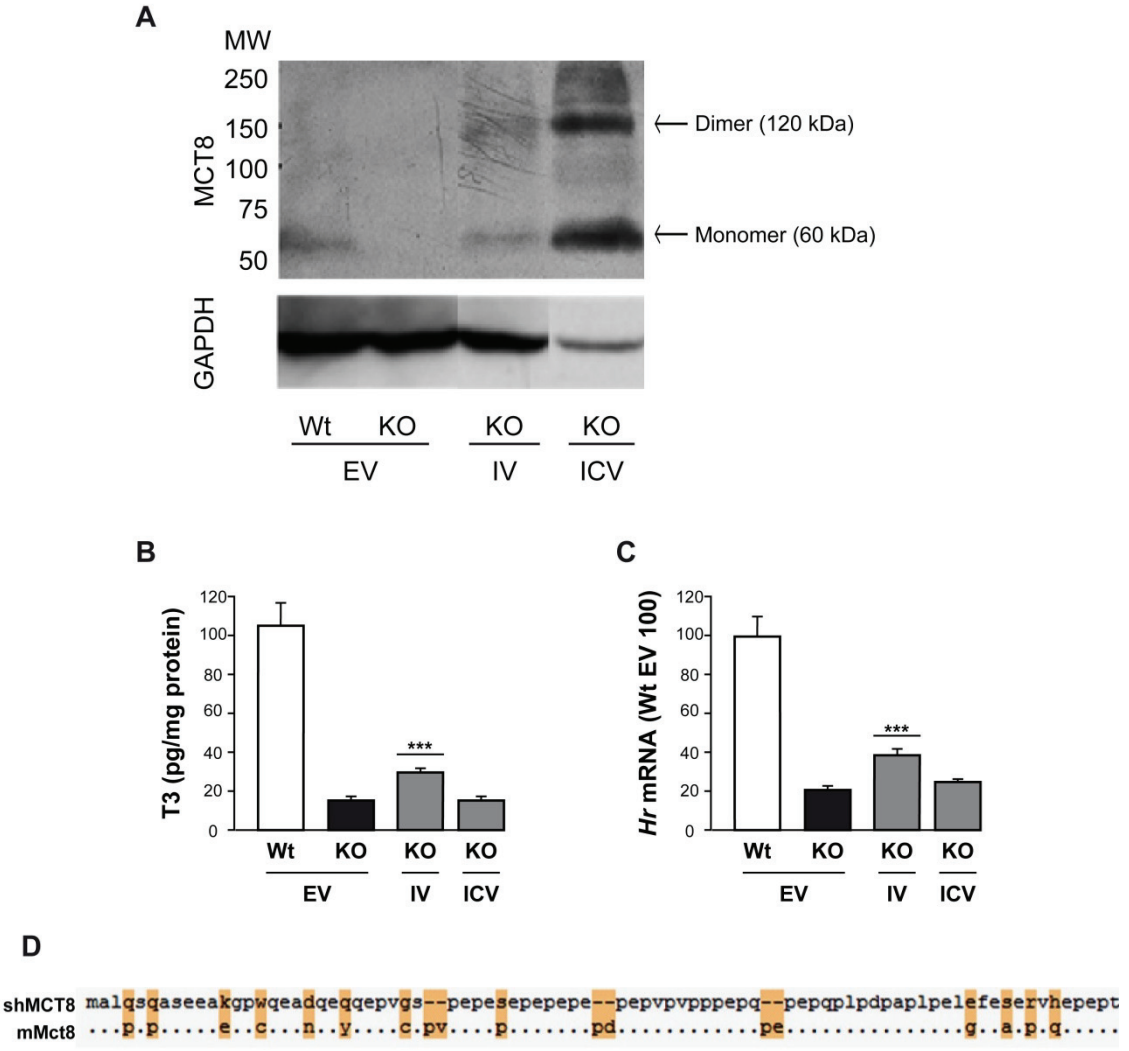


Figure 18. **hMCT8 protein in cerebrum of mice** injected with AAV9-ShMCT8 or empty vector (EV) intravenously (IV) or intracerebroventricularly (ICV). **(A)** Western blot analysis after SDS-PAGE of cerebrum homogenates developed with a hMCT8 antibody using GAPDH as a loading control. B and C show the effect of AAV9-ShMCT8 on T3 content **(B)** and induction of the T3-dependent gene *Hr* **(C)** in the cerebrum of IV and ICV injected mice. Data are expressed as means ± SE. Statistical differences for *Mct8*KO animals injected with ShMCT8 are compared to the same genotype given EV, ***p < 0.001. **(D)** Alignment of short human MCT8 (shMCT8) and mouse Mct8 (mMct8) along the peptide sequence that recognises the hMCT8 antibody. *Modified from Iwayama et al., 2016.*

2.2.2 hMCT8 protein localisation in brain of AAV9-ShMCT8 treated mice

To explore why delivery of hMCT8 presented some degree of functionality when it was given IV while this did not occur when it was delivered ICV, and especially taking into account that this last administration route provided more hMCT8 protein, the protein localisation was studied by immunohistochemistry. Immunohistological studies were performed at the Instituto de Investigaciones Biomédicas in Madrid and are part of this doctoral thesis.

For this, brains of perfused mice were harvested, sectioned and immunolabeled with a hMCT8 antibody which partially reacts with mouse Mct8 (**Figure 18 D**). In the Wt mice injected with EV, constitutive Mct8 protein was faintly detected in the cerebral cortex and cerebellum (**Figure 19 A, B**) and it was clearly detected in the choroid plexus (**Figure 19 C**). This is probably due to the partial immunoreactivity of the hMCT8 antibody with mouse Mct8 that stains regions with very high expression of the mouse Mct8, such as the choroid plexus. The *Mct8*KO animals injected with EV were labelled to a lesser degree (**Figure 19 E, F**) than the Wt injected with EV. As the *Mct8*KO mice are generated by deleting exon 3 it is likely that they synthesise the amino terminal fraction of the transporter producing a faint staining in cerebral cortex and the Purkinje cells of *Mct8*KO mice when using an antibody directed to the amino terminus of the molecule, as it is the case for this study. The positive immunolabelling signal detected in the choroid plexus of the Wt (**Figure 19 C**) was not present in the *Mct8*KO mouse (**Figure 19 G**), both given EV.

After delivery of AAV9-ShMCT8 through both IV and ICV administration in *Mct8*KO mouse, the hMCT8 protein was clearly present in the cerebral cortex (**Figure 19 I, M**), cerebellum (**Figure 19 J, N**), and choroid plexus (**Figures 19 K, O**). In agreement with results from Western blots, ICV injection of ShMCT8 produced greater amounts of the hMCT8 protein in cerebral cortex (compare **Figures 19 I, M; 20 J,N; 20 K,O**), compared to IV injection, and this hMCT8 protein appeared as aggregated deposits (**Figure 19 M**). Both in the IV and ICV injected mice, hMCT8 expression was predominantly in the cerebral cortex at the granular and infragranular layers of the neocortex and at the CA regions of the hippocampus (**Figure 20**). Subcortical regions presented few immunopositive cells, and dentate gyrus displayed almost lack of hMCT8 expression (**Figure 20 K, O**). Furthermore, the dendritic arborizations of some Purkinje cells in the cerebellum of both IV and ICV-injected mice were strongly labelled (**Figure 19 J, N**). The most important finding was the robust expression of hMCT8 in the choroid plexus of *Mct8*KO mice following IV-injected AAV9-ShMCT8 (**Figure 19 K**).

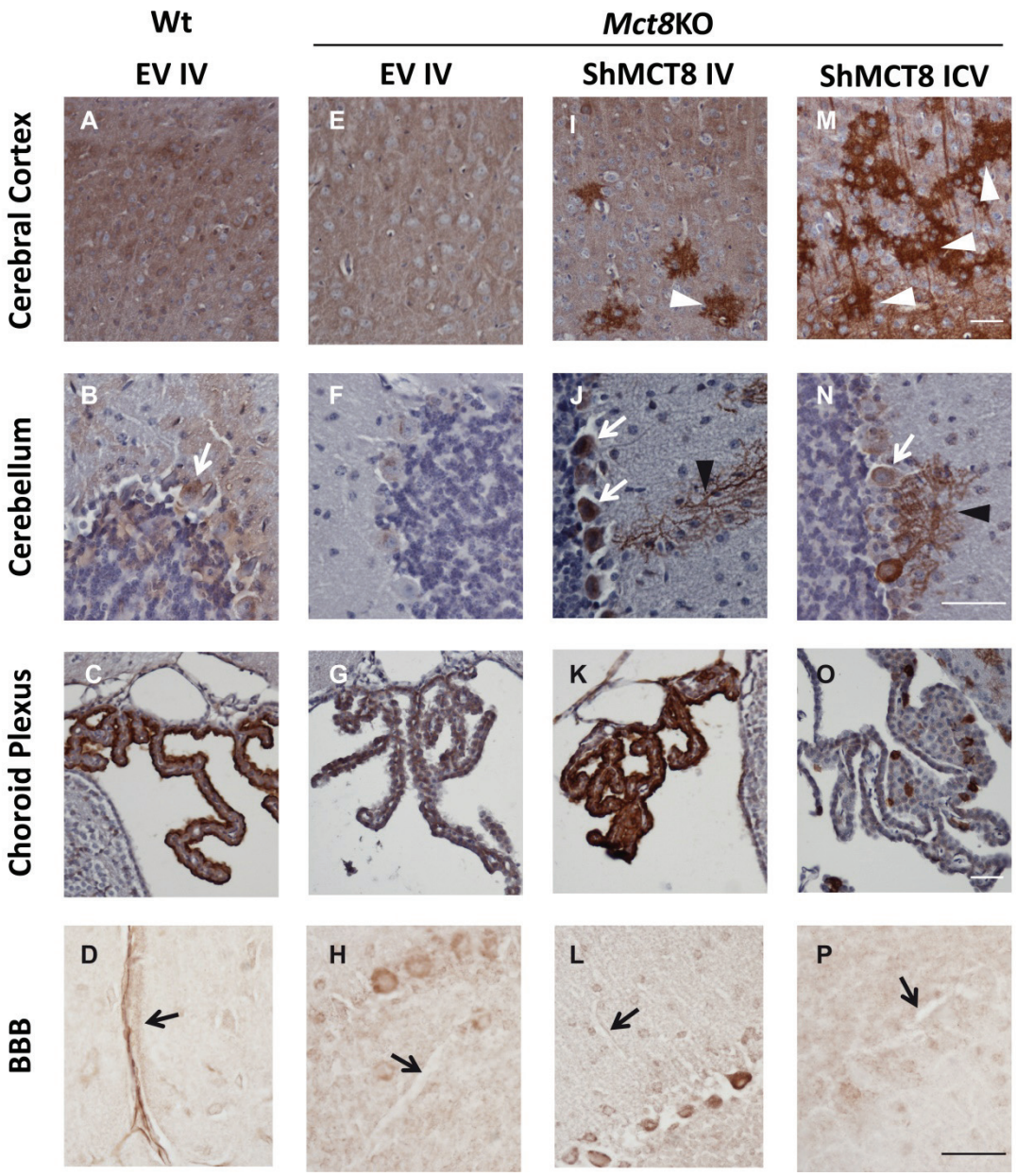


Figure 19. Localisation of the expressed hMCT8 protein in brains of *Mct8*KO mice given AAV9-ShMCT8 IV and ICV compared with Wt and *Mct8*KO animals injected IV with EV. Representative images showing hMCT8 expression (in brown) detected with a specific hMCT8 antibody in the somatosensory region of the cerebral cortex **(A, E, I, M)**, the cerebellar lobule 4 **(B, F, J, N)**, the choroid plexus **(C, G, K, O)** and the BBB in the cerebellar lobule 4 without hematoxylyn counterstaining **(D, H, L, P)** of Wt **(A–D)** and *Mct8*KO **(E–H)** mice injected with EV, injected with AAV9-ShMCT8 IV **(I–L)**, or ICV **(M–P)**. In agreement with Western blots results, hMCT8 was present in larger quantities in the cerebral cortex of mice injected with the virus ICV **(M)** than IV **(I)**. However, much of the immunoreactivity was present in aggregates (white arrowheads). Some Purkinje cells (white arrows) were observed in both IV- and ICV-injected cerebellum **(J, N)** with outstanding hMCT8 expression at the dendritic arborisations (black arrowheads). The control, Wt mice injected with EV, did not show the positive signal of dendritic arborisations **(B)**. hMCT8 was abundantly present at the choroid plexus of IV-injected *Mct8*KO mice **(K)** and only spottily expressed in mice given the virus ICV **(O)**. Positive immunolabelling was detected at the BBB of Wt mice injected with EV **(D)** and this labelling was absent in *Mct8*KO animals injected with EV **(H)** or ShMCT8 **(L, P)**. Scale bars 50 μm.

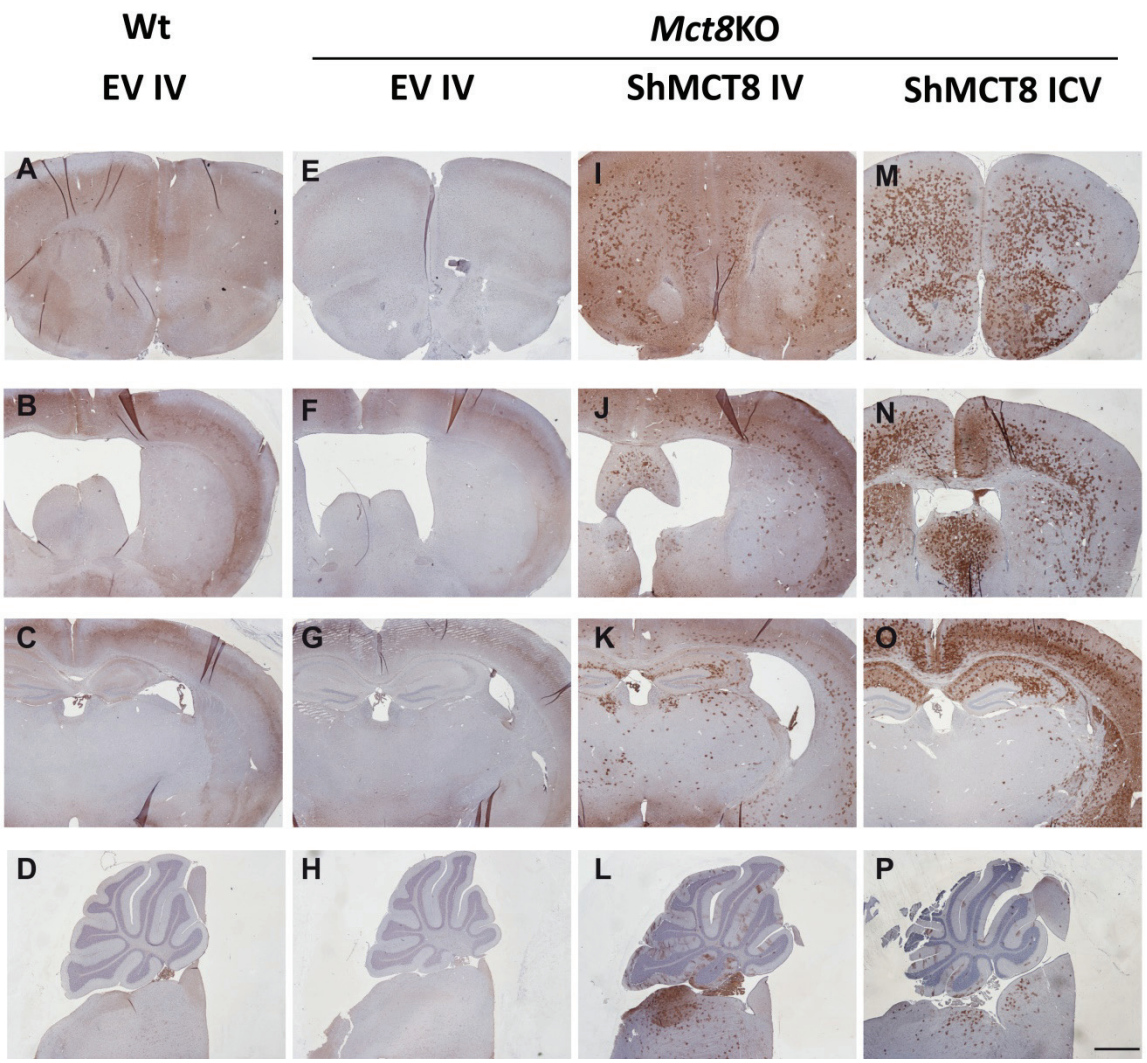


Figure 20. Panoramic views of the localisation of the expressed hMCT8 protein in brains of *Mct8*KO mice injected with AAV9-ShMCT8 IV and ICV compared with Wt and *Mct8*KO animals injected IV with EV. Sections A – O were taken on the coronal plane at the levels of Bregma 2.10 mm **(A, E, I, M)**; Bregma -0.22 mm **(B, F, J, N)** and Bregma -1.46 **(C, G, K, O)**. Cerebellar sections **(D, H, L, P)** were taken on the sagittal plane at the levels of Lateral 1.60 mm. Coordinates have been determined according to Franklin and Paxinos (2007). Scale bar 1000 µm.

Notably, the distribution of ShMCT8 after IV delivery was like the endogenous Mct8 observed in the Wt mouse (**Figure 19 C, K**) while ICV-delivered ShMCT8 was minimal and confined to a few cells of the choroid plexus (**Figure 19 O**).

Big efforts were put into determining whether hMCT8 was delivered to the BBB as well as to the choroid plexus. Under these experimental conditions the BBB was positively immunolabelled in the Wt animals within structures with low amount of neuropil such as the thalamus or the cerebellum (**Figure 19 D**). In the *Mct8*KO injected with EV animals the BBB did not present immunopositive signal in any region (**Figure 19 H**) and this was also the case

for AAV9-ShMCT8 IV and ICV-injected animals (Figure 19 L, P), suggesting that neither administration route led to hMCT8 expression at the BBB.

Finally, confocal microscopy studies verified that hMCT8 was targeted to the apical membrane of epithelial cells of the choroid plexus in IV-injected *Mct8*KO mice and only to a few cells of the choroid plexus in ICV-injected *Mct8*KO (Figure 21 A, B). Moreover, hMCT8 was targeted to the cell membrane of brain cells in both IV and ICV-injected mice (Figure 21 C-F).

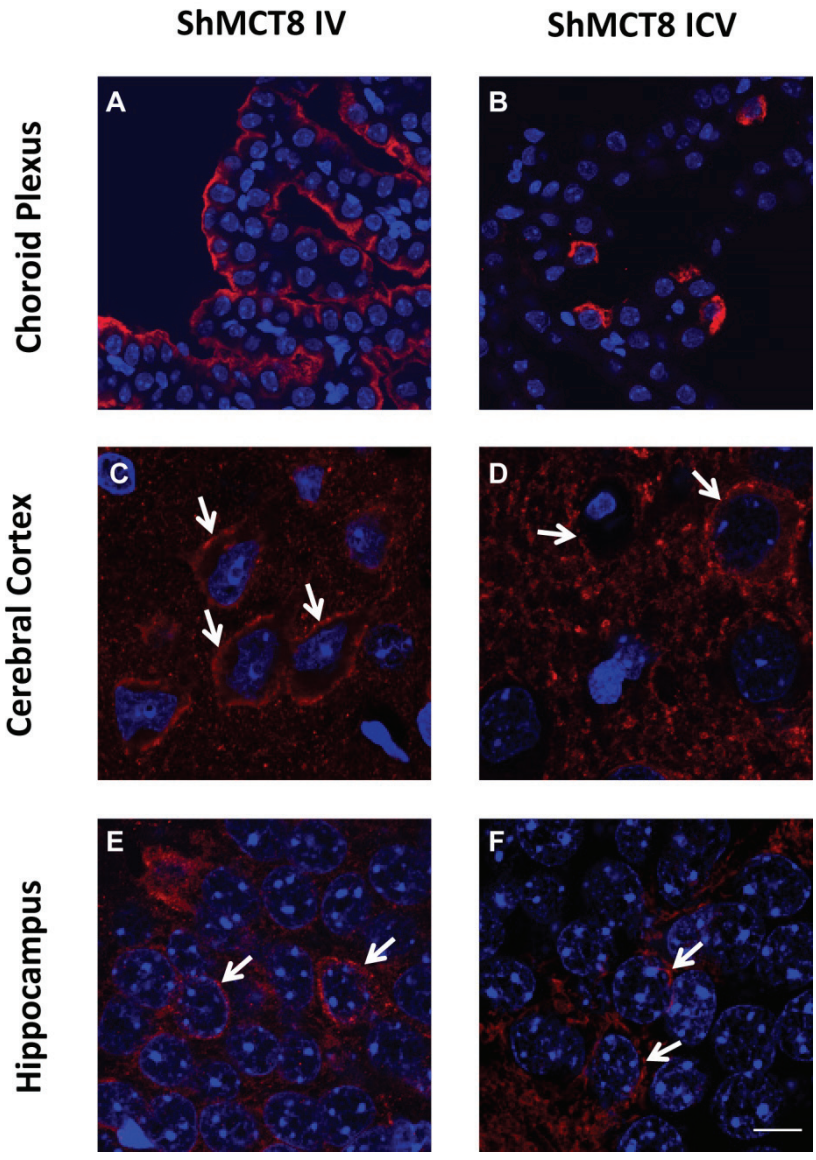


Figure 21. **Membrane localisation of hMCT8 in *Mct8*KO mice** injected with AAV9-ShMCT8 IV and ICV. Representative images showing hMCT8 (in red) and nuclei stained with DAPI (in blue) in the choroid plexus **(A, B)**, cerebral cortex **(C, D)** and CA1 region of the hippocampus **(E, F)** of *Mct8*KO mice injected with AAV9-ShMCT8 IV **(A, C, E)** or ICV **(B, D, F)**. hMCT8 was localised at the apical membrane of the choroid plexus of IV-injected mice **(A)** and only in a few cells of ICV injected animals **(B)**. hMCT8 was localised in the membranes (white arrows) of cortical and hippocampal brain cells of animals injected either IV or ICV **(C–F)**. However, a greater amount of hMCT8 was present in the cytoplasm of animals injected ICV. **(A, B)** Scale bar 20 µm; **(C–F)** scale bar 10 µm.

2.3 Novel strategies for thyroid hormone brain delivery: Intranasal route

2.3.1 Background

The use of delivery pathways that bypass the BBB has potential benefits for the treatment of the neurological condition of MCT8 deficient patients. The nasal cavity provides a direct pathway to the brain that can be used to deliver therapeutic agents by intranasal administration bypassing the BBB in a non-invasive fashion (Hanson and Frey, 2008). Therapeutics administered in the nasal cavity can reach the brain directly through the olfactory (Chen et al., 1998) or the trigeminal pathways (Thorne et al., 2004) as these pathways connect the nasal mucosa and the brain. The goal was to explore the use of intranasal delivery as a potential route to administer TH to palliate the hypothyroid conditions of *Mct8*KO mice in brain without worsening the peripheral hyperthyroidism.

Typically, drug formulations used for intranasal delivery must be highly concentrated as usually less than 1% of an administered drug can be nasally transported to the brain. High concentrated T3 and T4 formulations were design taking into account the solubility of TH and the requirements for intranasal delivery.

2.3.2 Exploratory studies

In the first place, exploratory studies were designed to quickly evaluate the potential use of the intranasal pathway for TH delivery in *Mct8*KO mice. Note that the number of animals used in these studies was not sufficient to obtain statistically significant results (2-5 animals); nevertheless the qualitative results were consistent enough to withdraw solid conclusions.

Initially, treatment with T4 was chosen over treatment with T3 as D2 activity can ensure an adequate delivery of T3 to the brain from T4 content. In a first set of experiments, Wt and *Mct8*KO mice were treated daily with T4 by intranasal delivery for a long-term period. After treatment, both Wt and *Mct8*KO mice exhibited a decrease in D2 activity in the cerebral cortex and increased expression of the T3-dependent gene *Aldh1a1* in the striatum, indicating an effect of T4 treatment in the brain (**Figure 22 A, B**). However, T4 and T3 plasma levels, as well as the expression of *Dio1* in the liver, were also increased after treatment (**Figure 22 C, D, E**) evidencing that intranasal delivery of T4 reaches the circulation. Subsequently, Wt and *Mct8*KO mice were treated with T4 by intranasal delivery for a short-term period and blood

was collected to assess T4 levels after treatment. T4 plasma levels were increased even after a short-term treatment (Figure 22 F).

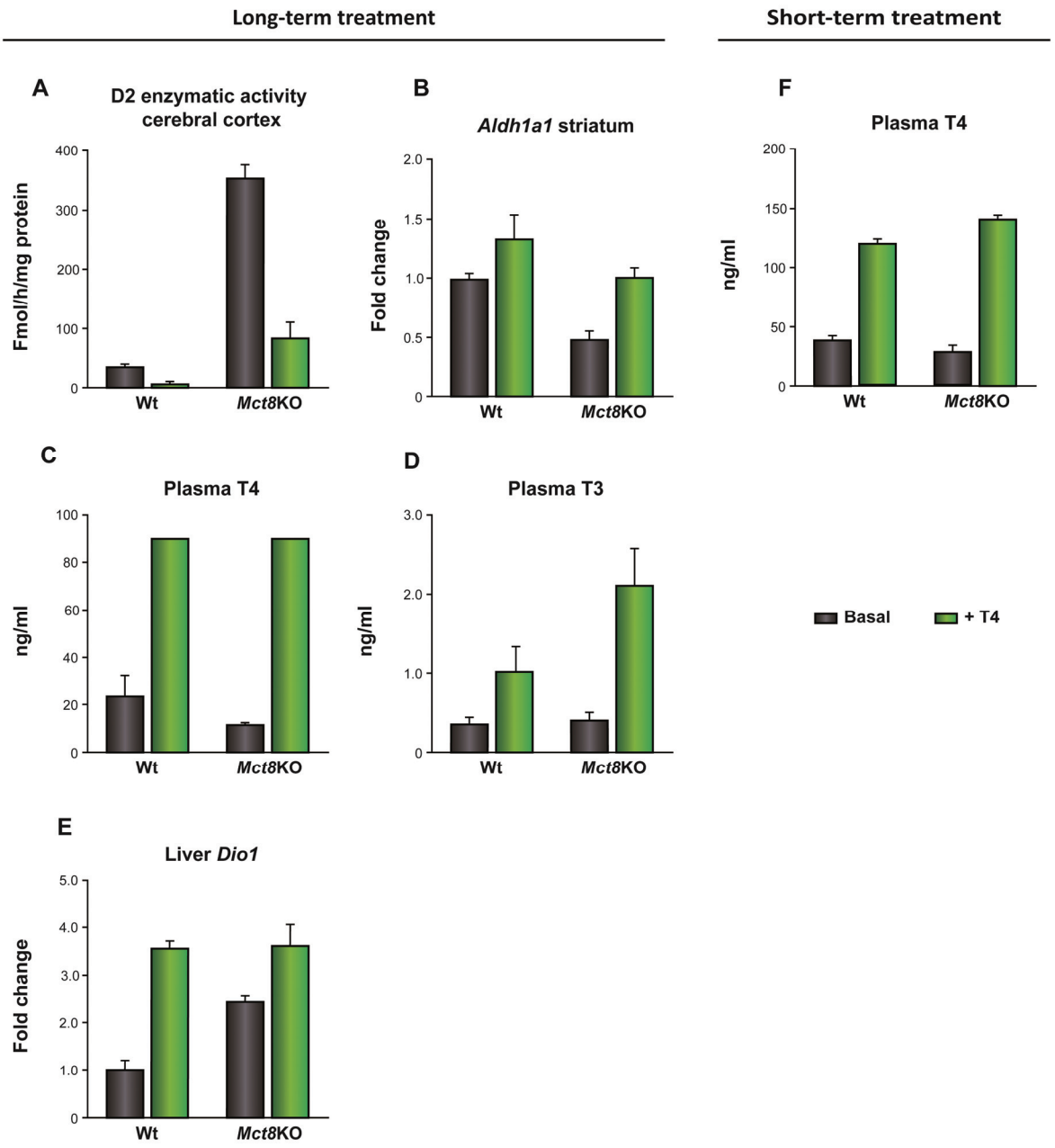


Figure 22. Effect of intranasal delivery of T4 to Wt and *Mct8*KO mice for a long-term period **(A – E)** or a short-term period **(F)**. **(A)** D2 enzymatic activity in the cerebral cortex and **(B)** gene expression analysis of the T3-dependent gene *Aldh1a1* in the striatum of Wt and *Mct8*KO mice in basal conditions and long-term T4 treatment. T4 **(C)** and T3 **(D)** plasma levels and gene expression of *Dio1* in liver **(E)** in Wt and *Mct8*KO mice in basal conditions and after long-term T4 treatment. **(F)** T4 plasma levels in Wt and *Mct8*KO mice in basal conditions and after short-term T4 treatment. As a preliminary test, no statistical analyses were performed due to the insufficient number of animals.

As mice unlike humans present the T4 transporter Oatp1c1 at the BBB it is not possible to differentiate if the effects of T4 treatment observed in the brain were due to direct access of T4 to the brain or were mediated through T4 from the circulation crossing the BBB. Furthermore, as MCT8 deficient subjects present high T3 serum levels, TH reaching the circulation should be avoided.

2.3.3 T3-BSA binding assay

In order to prevent TH from reaching the blood torrent after intranasal administration, the possibility of using carriers suitable for intranasal administration and capable of binding TH was evaluated.

Previous studies have shown that intranasal administration of BSA is able to distribute throughout mice brain shortly after delivery with only 1.5 – 4 % of the dose reaching the systemic circulation (Falcone et al., 2014). BSA is able to bind T3 with a high affinity (Okabe et al., 1975), which offers the possibility of using BSA as a carrier to deliver T3 to the brain through intranasal delivery without entering the blood stream.

As the drugs must be highly concentrated in order to be suitable for intranasal administration, the appropriate concentration of BSA that is able to bind a high concentrated solution of T3 was determined by a radioactive T3-BSA binding assay.

Two different concentrations of T3, one highly concentrated and a T3 replacement dose of 0.02 µg/µl (Di Cosmo et al., 2009) were incubated along with a known quantity of ^{125}I-T3 in presence of different concentrations of BSA: 0.25%, 0.5%, 1%, 1.5%, 2% and 5%. As shown in **Table 1**, the ability of BSA to bind T3 was similar for the high concentrated and the replacement dose solution. 1% BSA was already binding 84.1% of the high concentrated T3 and concentrating BSA up to 5% only increased T3 binding in 6% in comparison to the binding achieved by 1% BSA.

As the experiments assessing the distribution of BSA throughout the brain and the blood stream after intranasal administration were performed with 1% BSA (Falcone et al., 2014) and taking into account the outcome of the present T3-BSA binding assay, 1% BSA was considered the most suitable dose to use as a carrier for intranasal administration of T3.

	0.25% BSA	0.5% BSA	1% BSA	1.5% BSA	2% BSA	5% BSA
High T3	70.5%	79.7%	84.1%	87.8%	88.06%	90%
Replacement T3 (0.02µg/µl)	ND	79.9%	88.8%	ND	94.7%	97.2%

Table 1. Percentage of two solutions of T3 (one highly concentrated "High T3" and a T3 replacement dose "Replacement T3") bound to different concentrations of bovine serum albumin (BSA). ND = not determined.

2.3.4 Intranasal T3 treatment

Finally, the ability of T3 to reach the mouse brain circumventing the blood was assessed by treating Wt mice with a high concentrated solution of T3 bound to 1%. Furthermore, the capacity of the vasoconstrictor PHE (Dhuria et al., 2009) to reduce the passage of the administered solution to the circulation was also evaluated. The following groups were prepared: Basal Wt; Wt + T3 (Wt treated with T3); Wt + T3-BSA (Wt treated with T3 bound to 1% BSA); Wt + T3-BSA-PHE (Wt treated with T3 bound to 1% BSA and PHE 1%). Following a short-term intranasal treatment, blood was collected to assess T3 plasma levels.

T3 determinations in plasma revealed that intranasal treatment with T3 reached the circulation as expected and increased T3 plasma levels about 10 times in comparison to the Wt. Intranasal delivery of T3 bound to BSA alone or in combination with the vasoconstrictor PHE was not able to prevent T3 from reaching the circulation as both treatments increased T3 levels in plasma even more than 10-fold the levels of the Wt (**Figure 23**).

The use of BSA or the vasoconstrictor PHE does not improve the TH intranasal delivery treatment as they do not prevent TH from reaching the blood torrent.

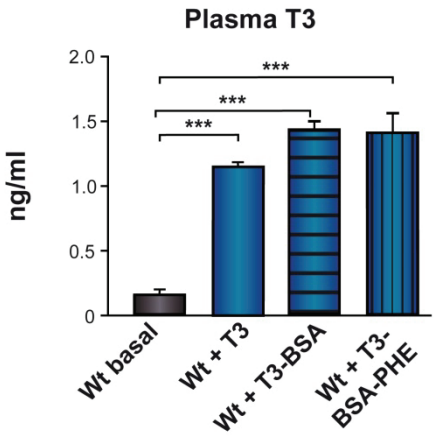


Figure 23. **T3 plasma levels in Wt mice after intranasal administration** of a high concentrated solution of T3 alone (Wt + T3), bound to 1% BSA (Wt + T3-BSA) or bound to 1% BSA and in presence of the vasoconstrictor phenylephrine (Wt + T3-BSA-PHE). Data are expressed as means ± SE. ***p < 0.001 was determined by One-way ANOVA and Bonferroni's post hoc test.

DISCUSSION

TH play an essential role both in the developing and the adult CNS. Deficit of TH leads to neurological abnormalities with different functional consequences depending on the timing and the cause for TH deficiency. The developing CNS is particularly sensitive to TH deficiency as TH deficit may cause irreversible damage. Understanding the physiological mechanisms that underlie T3 availability to the developing brain is essential to get insight into pathological conditions.

In the lasts years our group has focused on a rare disease caused by mutations in the TH transporter MCT8 known as the AHDS. Patients present severe neurological impairments caused by diffuse brain damage existent from prenatal stages, for which there is presently no effective treatment. Knowledge of TH availability and action during brain development will help to design therapeutics and timing of treatment for TH related diseases, namely for the AHDS.

The present work has aimed to: (i) explore the physiological mechanisms that ensure appropriate levels of T3 during perinatal stages of mouse brain development with special focus on the relative contribution of maternal TH and foetal brain D2 activity and (ii) to perform preclinical studies in Mct8 deficient mice to explore possible therapies for MCT8 deficient patients.

PART I: Contribution of maternal hormones and foetal D2 to thyroid hormone brain economy during perinatal development in mice

The concentrations of the genomically active hormone T3 in the brain depend on TH transport across the brain barriers, the local generation of T3 in astrocytes by D2, and TH degradation by D3. In addition, circulating foetal TH concentrations derive partly from the mother. How all these factors interact to ensure “brain euthyroidism” has not been studied in mice. The contribution of maternal TH to embryos has previously been extensively studied in rats, using models of combined maternal and foetal hypothyroidism (Calvo et al., 1990, Grijota-Martínez et al., 2011). In these studies T4 administration to hypothyroid pregnant dams resulted in an increase of foetal T3 brain content along with an increase in the expression of T3-dependent genes. On the contrary, T3 administration to hypothyroid pregnant dams did not increase foetal T3 brain content and did not induce the expression of T3-dependent genes despite the expression of the TH transporter Mct8 at the BBB and even in presence of T3 in foetal plasma

and tissues other than the brain. It was therefore proposed that foetal brain is crucially dependent on T4.

Traditionally the rat has been the most commonly used animal model to study the thyroid physiology and TH action in brain. However, in the last years the mouse has replaced the rat as the most widely employed model due to the growing accessibility to genetically modified mice and this is providing new insights about thyroid physiology and pathological events. As pointed out above, TH brain availability during mouse brain development has not yet been studied. Detailed knowledge of TH availability and economy during mouse embryonic development is essential to understand the changes induced by genetic manipulations of transporters and deiodinases.

The aim of the present study was to evaluate the relative contribution of maternal TH and foetal brain D2 to TH foetal brain homeostasis in mice by treating euthyroid pregnant dams with T4 or T3 from E12 to E18. Due to the difficulties to obtain plasma samples from E18 foetuses, foetal livers were used assuming that TH content in the liver reflects TH levels in plasma.

1.1 Peripheral effect of thyroid hormone treatment in dams and foetuses

After T3 treatment, T4 decreased in maternal plasma, in all probability caused by TSH suppression, and it also decreased in foetal liver. T3 was increased in maternal plasma, but not in foetal liver. As maternal T3 treatment has been shown not to suppress foetal TSH (Morreale de Escobar et al., 1993, Grijota-Martínez et al., 2011), the decrease in the foetal liver T4 would reflect the reduction of circulating maternal T4. In view of this, a fraction of at least 50% of total liver T4 in the E18 mouse foetus derives directly from the mother. After T4 treatment, T4 was accordingly increased in the maternal plasma and also in the foetal liver, confirming that exogenously administered T4 to the mothers can cross the placental barrier (Dong et al., 2015). In contrast T3, which would be derived from monodeiodination of the administered T4, was increased in maternal plasma but again not in foetal liver. These results suggest that the exogenously administered T3 and the endogenously produced T3 from the administered T4 to the mother had similar distributions in the maternal and foetal compartments.

Interestingly, even though T4 was increased in the foetal liver after administration to the mothers, T3 was not increased, suggesting lack of foetal T4 to T3 conversion in liver or elsewhere. Ontogeny studies in rats (Bates et al., 1999) showed that D1 activity and *Dio1*

mRNA were detectable in several foetal tissues at E21, but at very low levels. Low tissue activity of D1, together with a high Km for T4 would predict negligible 5' deiodination of T4. This is in agreement with studies in mouse foetal liver with undetectable levels of *Dio1* mRNA expression (Li et al., 2012) and D1 activity (Ferrara et al., 2013). In the present study, D1 activity was not detectable in the liver of euthyroid E18 foetuses and, moreover it was not responsive to variations in the liver TH content resulting from T3 or T4 treatment to the mothers.

A first conclusion is that a large amount of foetal T4 could be of maternal origin and that maternal-foetal T3 transfer is tightly regulated most probably at the placental level. This contrasts with findings in rats in which decreased levels of T4 in thyroidectomised mothers did not result in decreased T4 content in foetal liver (Grijota-Martínez et al., 2011). These discrepancies might be explained by the timing of thyroid gland development and the moment in which the determinations were made. In the experiments with rats, determinations were made on E21, 4 days after onset of foetal thyroid function. In the current mouse experiments, determinations were made at E18, i.e., 1.5 days after onset of foetal thyroid function. Therefore the contribution of the foetal thyroid would be larger in the rat experiments than in the present studies. As a matter of fact, in the E18 mouse the contribution of maternal T4 can be estimated to be above 2/3 of the total foetal liver T4 concentration from the results in **figure 6B**.

One of the limitations of the present study is that the levels of TH in foetal plasma are being inferred from the TH content detected in the foetal liver. Expression of transporters *Lat1* and *Lat2* was downregulated in the foetal liver after treatment which opens up the possibility that foetal TH liver content might not be accurately reflecting the plasma levels. However, taking into account that concentration of circulating amino acids is far higher than that of TH, it is difficult to make any conclusions about the physiological significance of this downregulation on TH transport as it might be reflecting a decrease in the need for amino acids in the liver. Moreover, past studies have proven accurate correlations between foetal TH content in peripheral tissues like liver and heart with TH levels in plasma (Calvo et al., 1990). Furthermore, the main TH transporter in the liver, NTCP, and the Mct8 transporter are not differentially regulated in hyperthyroid conditions (Engels et al., 2015), suggesting that TH transport to the liver is not impaired under hyperthyroid conditions.

1.2 Effects of maternal thyroid hormone treatment in foetal brain

Regarding the foetal brain, T4 content reflected the peripheral T4 status of the foetus while T3 content remained constant under a range of T4 concentrations in brain. As for the liver, it appears that a fraction of at least 50% of total brain T4 in the E18 mouse foetus derives from the mother. In accordance to the stable concentrations of T3 in the foetal brain was the expression of the T3-dependent gene *Aldh1a1* which was constant despite the variations on the T4 brain content. T3 brain homeostasis was not achieved through the regulation of TH-transporters as the expression of *Mct8*, *Oatp1c1* or *Lat2* did not vary in consequence to the treatments, that is to say, their expression did not vary under changing concentrations of brain T4. The relative expression of these transporters at embryonic stages in comparison to later stages in life such as P30 revealed that *Mct8* expression is the same at both ages whereas *Oatp1c1* and *Lat2* expression is higher at P30. In accordance with this, *Mct8* expression at E18 was much more abundant than *Oatp1c1* and *Lat2* expression in foetal brain. This could be pointing to the importance of Mct8 during embryonic development although its role still remains unknown.

Another way to achieve brain TH homeostasis is through the regulation of deiodinases. D3 is necessary for the regulation of T3 brain content and it is particularly important during development (Hernandez et al., 2010, Zoeller, 2010). qPCR results demonstrated that *Dio3* expression levels in the foetal brain were not modified as a result of the different treatments. As *Dio3* mRNA content and D3 activity are well correlated the results suggest that, under these circumstances, D3 is not involved in facilitating an appropriate content of T3 in the foetal brain. Expression of *Dio3* in neonatal stages of development might be limited to specific regions, such as to areas involved in sexual differentiation, to control local T3 concentrations, as has been described in rats (Escámez et al., 1999). On the other hand, D2 activity in the foetal brain was elevated under reduced T4 content and decreased under elevated T4 brain content suggesting that D2 activity plays a critical role in maintaining an appropriate content of T3 in the foetal brain. *Dio2* mRNA expression analysis revealed no changes at the transcriptional level despite the different treatments. *Dio2* is regulated at transcriptional level by its end product T3, whereas its substrate T4 regulates its activity at post-translational level (Burmeister et al., 1997) so the results overall reinforce that brain T3 content is not altered despite the different treatments, that the treatments alter T4 brain content and that T3 derived from T4 by D2 activity in the brain provides an essential source of T3 during mouse foetal development.

Detection of *Dio2* mRNA expression by *in situ* hybridization at E15 suggests a role for TH in early development before the onset on the foetal thyroid gland, that in the mice is E16.5. *Dio2* mRNA expression greatly increases at E18, the day prior to birth as it has been reported in rat activity assays (Ruiz de Oña et al., 1988). At these stages, detection of *Dio2* mRNA expression at the meninges, the ependymal layer of the lateral ventricles and the choroid plexus suggests an important role of D2 at the BCSFB. *Dio2* mRNA expression at the meninges is of great importance as the primitive meninge is the origin of the vascular plexus that will give rise to the brain vasculature (Marin-Padilla, 1985). *Dio2* mRNA expression at the ependymal layer of the lateral ventricles and the choroid plexus is also highly relevant as at these stages the lateral ventricles take up a big surface of the brain and are largely occupied by the choroid plexus (O'Rahilly and Müller, 2008) and because the choroid plexus may have a greater transport capacity during early stages of brain growth and development (Keep and Jones, 1990).

So during brain angiogenesis and brain embryonic development, T4 transport across the choroid plexus along with D2 activity transforming T4 into T3 at the BCSFB might be key events of TH action in brain in contrast to adult stages when T4-to-T3 conversion by D2 is mediated mostly in the astrocytes (Guadaño-Ferraz et al., 1997). Furthermore, TH at perinatal stages might be regulating key events such as corticogenesis in the meninges (Decimo et al., 2012) or embryonic neurogenesis in the lateral ventricles (Urban and Guillemot, 2014).

It is important to point out that the data suggest that, although maternal T4 contributes enormously to the total foetal T4 pool, under low levels of maternal T4 the foetus is able to compensate for the lack of maternal TH. This is also observed in foetuses coming from thyroidectomised rats that did not present alterations in the T3 or T4 brain content, in the expression of T3-dependent genes (Grijota-Martínez et al., 2011), or in the brain D2 activity (Ruiz de Oña et al., 1988) suggesting an important role of the foetal thyroid gland in rats. In the present study, a reduction in the plasma levels of T4 in the mother led to a decrease in T4 foetal brain content and consequently to an increased D2 activity highlighting important regulatory mechanisms in the foetal brain. This might indicate that in absence of maternal TH, the foetus can trigger compensatory mechanisms, and conversely, when the foetal gland is not functional (e.g. after treatment with antithyroid drugs) maternal TH can compensate for the lack of foetal TH. Nevertheless, a previous study demonstrated that administration of T4 to mothers increased the expression of T3-dependent genes in the cerebral cortex of E16 foetuses (Dong et al., 2015) suggesting that during early developmental stages foetuses might be more sensitive to maternal TH.

1.3 Role of D2 in Mct8 deficiency during early brain development

After proving for the first time the important role that D2 plays during mouse brain embryonic development in the supply of brain T3, its contribution to perinatal brain development was further evaluated by using different KO mice models. The expression of T3-dependent genes was analysed as an indicator of the brain TH status under D2 deficiency conditions. The contribution of D2 to the TH brain excess characteristic of perinatal *Mct8*KO mice was also evaluated.

This was done by comparing the expression levels of a set of T3-responsive genes (*Hr*, *Shh* and *Klf9*) in Wt, *Mct8*KO, *Dio2*KO and double KO *Mct8/Dio2*KO mice in the cerebral cortex at P3. In *Mct8*KO animals the expression of *Hr* and *Shh* was increased in comparison to Wt animals. This was indicative of a situation of brain hyperthyroidism in *Mct8*KO mice as already known (Ferrara et al., 2013, Nuñez et al., 2014). *Dio2*KO animals presented brain hypothyroidism with reduced expression of *Hr* and *Shh*. *Klf9* presented mild increased expression in *Mct8*KO animals and subtle decrease in *Dio2*KO mice, not statistically significant in both cases, indicating that at these stages *Klf9* might still not be fully sensitive to TH. The hypothyroid brain condition present in *Dio2*KO mice at P3 did not trigger an increase in the expression of the gene encoding for Mct8 as a compensatory mechanism. Brain hypothyroidism, with reduced expression of T3-dependent genes during perinatal stages of *Dio2*KO mice contrasts with *Dio2*KO mice at P15 when the expression of T3-responsive genes is not altered (Galton et al., 2007) and reinforces the findings above indicating that D2 plays a critical role in the supply of T3 during perinatal brain development.

Expression of *Hr*, *Shh* and *Klf9* in the double *Mct8/Dio2*KO mice was the same as in the single *Dio2*KO indicating that *Mct8/Dio2*KO animals present a state of brain hypothyroidism. It can be concluded that D2 activity is necessary to generate the hyperthyroid state in the brain of *Mct8*KO animals during perinatal stages of development and that TH excess is not due to increased TH transport directly from the systemic circulation. During perinatal life *Mct8*KO mice manifest slight hyperthyroxinemia. This could provide a relative excess of substrate to the D2, sufficient to increase T3 formation but not to increase D2 degradation. It is clear that D2 provides the brain with T3; however, the role of Mct8 transporter at these stages of development still remains unknown and it cannot be ruled out that Mct8 mediates T3 efflux and/or degradation of T3 by D3, as suggested previously (Nuñez et al., 2014, Stohn et al., 2016).

The fact that the expression of T3-dependent genes in the double *Mct8/Dio2*KO animals was not different from the hypothyroid state present in the single *Dio2*KO mice suggests that D2 might be only or most relevant source of brain T3 during perinatal development.

Altogether the data suggests that during mouse prenatal brain development maternal T4 is an essential source of TH. T4 reaches the CSF in the meninges and the lateral ventricles, where D2 is present, through the choroid plexus so T3 could be locally formed. Taking into account the large surface of the ventricles at these stages, T3 could reach the brain through the ventricles and the meninges and access the neural cells by TH transporters to exert its action at the genomic level. *Dio2* mRNA expression in other brain structures different from the BCSFB, such as the striatum, suggests that D2 in the astrocytes may be already generating T3 through T4 available from the BBB although probably in a lesser extent than in the adult (**Figure 24**).

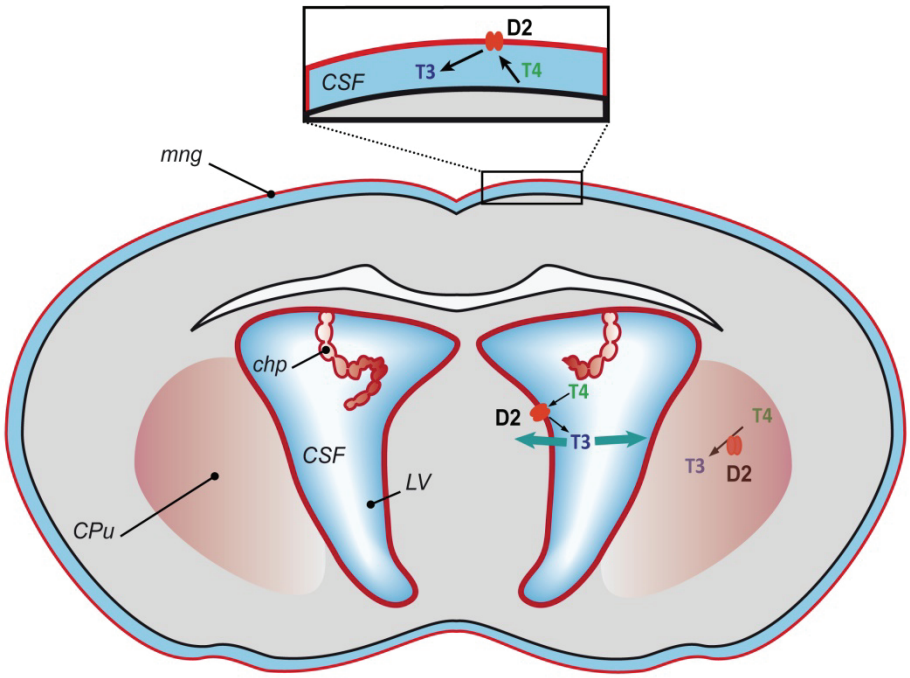


Figure 24. **Proposed model of T3 availability to the mouse developing brain**. T4 mostly of maternal origin reaches the brain mainly through the CSF and gets converted into T3 by D2 (in red) at the BCSFB (mng, LV, chp). From the CSF the T3 could spread throughout the brain and access the neural cells to exert its action. *Dio2* mRNA expression at other structures such as the CPu suggests that T4 can also cross the BBB and be locally converted into T3 by D2, although probably in a less important fashion than in adult mouse. chp, choroid plexus; CFS, cerebrospinal fluid; CPu, caudate putamen (striatum); LV, lateral ventricles and mng, meninges.

PART II: Therapeutic approaches for MCT8-deficiency treatment

MCT8 deficient patients present severe mental retardation, impaired ability to speak, diminished muscle tone and movement abnormalities. Currently there is no effective treatment to improve the neurological impairments of MCT8 deficient patients. Moreover, studies in rodents and humans suggest that the neurological defects are compatible with the deficient action of T3 in the developing brain caused by impaired TH availability to the target neural cells (López-Espíndola et al., 2014) and that the main restriction for T3 entry into the target neural cells is at the BBB (Ceballos et al., 2009). For this reason the therapeutic strategies developed in this thesis have focused on methods that can either bypass the brain barriers by the use of TH analogues that are not transported by Mct8, such as TRIAC, by the use of alternative delivery pathways that can circumvent the BBB, such as intranasal administration or by replacing MCT8 by the use of AAVs to try to correct brain defects.

2.1 Thyroid hormone analogues: TRIAC

TRIAC is a TH analogue with many known properties that make it a good candidate for the treatment of MCT8 deficiency. However, how therapeutic doses of TRIAC may affect the physiology of *Mct8*KO mice or whether it is able to exert a function at the genomic level in the brain, inducing similar neuronal gene responses as T3 *in vivo,* is not known. Although *Mct8*KO mice do not present gross neurological abnormalities, they faithfully replicate the endocrine abnormalities of patients as well as they display alterations in the expression of some T3-dependent genes that offer the possibility to assess TRIAC action in the brain. Hence, the goal was to characterise the effects of therapeutic doses of TRIAC in Wt and *Mct8*KO mice with special focus on the CNS of juvenile mice to ultimately evaluate TRIAC as a possible therapeutic agent suitable for patients with MCT8 mutations.

The treatment consisted on the administration of therapeutic doses of TRIAC (30 ng TRIAC/g BW) in the drinking water from P21 to P30 to Wt and *Mct8*KO littermate mice. Treatment greatly increased TRIAC plasma levels in both the Wt and *Mct8*KO animals similarly, suggesting that TRIAC availability and metabolism was not modified in the *Mct8*KO. TRIAC treatment drastically decreased plasma T4 levels in both the Wt- and *Mct8*KO-treated animals evidencing that Mct8 deficiency does not interfere with the inhibition of TSH by TRIAC. T4 plasma levels in *Mct8*KO animals after treatment were 6 times lower than those of basal Wt animals indicating that therapeutic doses of TRIAC lead to severe hypothyroxinemia, which

may be potentially harmful unless counteracted by its thyromimetic effect. One of the most relevant findings is that TRIAC treatment suppressed the elevated levels of T3 characteristic of Mct8 deficient animals, and even decreased it below normal levels. In contrast, it did not affect T3 plasma levels of Wt-treated animals. This is of great relevance as, in this respect, TRIAC could be a beneficial treatment in ameliorating the peripheral tissue hyperthyroidism present in *Mct8*KO animals. In the treated Wt animals, T3 plasma levels are likely maintained by the elevated D1 activity in the liver which is in turn stimulated by TRIAC regardless of the low T4 plasma levels. In the *Mct8*KO-treated mice, the further decreased levels of plasma T4 are not enough to maintain the high plasma T3 levels characteristic of this genotype despite the increase in D1 activity. This observation is consistent with the T4/T3 plasma ratios which are similar in the treated Wt and *Mct8*KO mice being 43.5 and 45.2, respectively.

It is already known that TRIAC enters the liver after treatment (Medina-Gomez et al., 2008). Like T3, TRIAC exerts a direct action in the liver as D1 activity and *Dio1* expression increased in the Wt and *Mct8*KO mice after treatment. Therefore, although TRIAC treatment restored the plasma T3 levels in *Mct8*KO animals into the reference range, the liver was still hyperthyroid, due to its thyromimetic action. On the other hand, at the dose used in this study, TRIAC did not affect the expression of the T3-dependent gene *Serca2a* in the heart. This is in agreement with two other separate studies in rodents, in which similar or even higher doses of TRIAC than those used in this study failed to increase *Dio1* mRNA abundance and D1 activity in the heart, despite clear uptake of TRIAC (Medina-Gomez et al., 2008, Liang et al., 1997) in the tissue.

Because patients suffering from MCT8 deficiency present very severe neurological impairments and the therapeutic options currently available have not been successful in improving these disorders, the ultimate goal of this study was to determine whether therapeutic doses of TRIAC are able to reach the brain and exert a thyromimetic action in the absence of Mct8.

In vitro studies have demonstrated that TRIAC has the ability to induce similar neuronal gene responses as T3 (Kersseboom et al., 2015). Unfortunately, the use of the established therapeutic doses *in vivo* failed to reproduce this effect in *Mct8*KO mice. TRIAC treatment did not increase the expression of T3-responsive genes neither in the cerebral cortex nor in the striatum and, on the contrary, there was a reduction in the expression of a few T3-dependent genes in both cerebral regions after treatment. This suggests that therapeutic doses of TRIAC are not sufficient to induce gene expression in the brain, and may aggravate the mild brain hypothyroid situation present in *Mct8*KO mice. This observation was confirmed by the direct

measures of TRIAC and T3 content in the cerebral cortex and the striatum of untreated Wt and *Mct8*KO animals and after TRIAC treatment. In the striatum TRIAC content did not increase after treatment with TRIAC in the Wt or the *Mct8*KO animals. In the cerebral cortex there was a subtle increase in the content of TRIAC in both Wt and *Mct8*KO animals after treatment, but this increase was not statistically significant. Regarding T3 content, there was a substantial decrease after TRIAC treatment in both genotypes on each cerebral region. These two observations provide direct evidence to support that this dose of TRIAC is not sufficient to increase TRIAC in the brain or to have effects on gene expression in brain cells and that it aggravates the hypothyroid state of the brain.

Because the main source of T3 in the brain is the one provided by the local conversion of T4 into T3 by D2 (Crantz et al., 1982), most probably the reduction in the T3 content of the brain was due to the great decrease in the plasma T4 levels of the treated animals, especially in the TRIAC-treated *Mct8*KO animals. TRIAC treatment did not have an effect on D2 activity in the cerebral cortex of either WT or *Mct8*KO mice in comparison to their basal controls. Other studies have already described that similar doses of TRIAC as the one used here do not affect D2 cerebral cortex activity in hypothyroid rats (Liang et al., 1997), suggesting that TRIAC does not have an effect *per se* on D2 brain activity.

It is concluded that treatment with therapeutic doses of 30 ng TRIAC/g BW/day in *Mct8*KO mice restores plasma T3 levels but severely decreases T4 levels. TRIAC treatment increases D1 activity and *Dio1* gene expression in the liver, but does not have any effect on cardiac T3-dependent gene expression. While TRIAC has a potential beneficial effect reducing the peripheral hyperthyroidism without affecting the heart, it is not able to normalise the thyrotoxic effect in the liver characteristic of Mct8 deficiency. Also, although treatment with the established therapeutic doses of TRIAC increased 3-fold the TRIAC levels in plasma; these were insufficient to increase TRIAC content in the brain and to promote the expression of T3-dependent genes in brain cells. Furthermore, treatment led to a situation of brain hypothyroidism with reduced T3 content. TRIAC treatment would generate a situation of severe hypothyroxinemia that could be potentially harmful, especially for the brain, suggesting that TRIAC treatment in MCT8 deficiency should be considered with caution.

2.2 Gene therapy: Adeno-associated virus

In the last years the use of AAVs has been increasingly successful in clinical gene therapy both for monogenic and complex diseases (Kotterman and Schaffer, 2014) and offers a potential treatment for patients suffering from MCT8-deficiency. Among the available AAV vectors, AAV9 has been shown to cross the BBB and to transduce wider brain areas and more cell types than other AAVs (Foust et al., 2009).

In this approach we aimed to evaluate the effects of the delivery of the short isoform of human MCT8 (ShMCT8) cDNA in the CNS of *Mct8*KO animals *in vivo* using AAV9 viral vectors. The AAV9-ShMCT8 constructs were administered IV or ICV into P1 *Mct8*KO mice. Interestingly, a significant and critical difference was observed in the functional effects of ShMCT8 when it was delivered by ICV as compared to IV injection. Despite the fact that the ICV route generated a higher level of transcription and translation of hMCT8, only injection through the IV route produced a functional effect, as both T3 brain content and expression of the T3-dependent gene, *Hr*, were increased after daily administration of T3 for 4 days before P28 when brains were collected.

When the delivered hMCT8 was studied at a immunohistochemical level it was observed that both IV and ICV injections of AAV9-ShMCT8 produced protein in the membrane of neural cells and within neuronal processes such as dendrites in cerebral cortex, hippocampus and cerebellum, with the ICV injection producing greater amounts of the hMCT8. The most important finding was at the choroid plexus where normal distribution of MCT8 at this structure was only achieved by IV administration of the AAV9-ShMCT8. This is of great relevance as the choroid plexus and the endothelial cells lining blood vessels mediate the entry of TH from blood and CSF into the brain. According to studies in rat, the choroid plexus is responsible for 20% of the TH brain content (Chanoine et al., 1992) which is in agreement with the magnitude of increase in brain T3 content in the current study. This observation along with the fact that neither IV nor ICV injection was able to induce hMCT8 expression at the BBB reinforces what it is already stablished: that MCT8 is essential at the BBB to mediate the entrance of TH to the brain (Ceballos et al., 2009). In this way, failure of full integration of hMCT8 into BBB could explain the partial rescue of T3 uptake in brains of IV injected *Mct8*KO mice compared to Wt controls.

Therefore, it is a logical conclusion that for gene therapy-delivered MCT8 to provide maximal function, the MCT8 must be targeted to the brain barriers as required for T3 entry into brain tissue. Additionally, it might be necessary or beneficial for MCT8 to reach the cell membrane

for TH transport into nervous system cells, although this is not the limiting step. It is therefore necessary to optimise gene isoform, delivery strategies and viral vector in order to achieve functional MCT8 within the brain. These are required to properly target MCT8 to the brain barriers using an AAV vector and must be critically considered for the development of a successful gene therapy for patients with MCT8 deficiency.

2.3 Novel TH delivery routes: Intranasal delivery

The last approach for the development of an effective treatment to address the neurological impairments of MCT8 deficient subjects consisted on the administration of a brain TH replacement therapy delivered through the nasal cavity, as this has the potential to bypass the BBB and reach the brain (Hanson and Frey, 2008). Intranasal delivery seemed an encouraging option as it has been proved successful to deliver insulin in patients with Alzheimer's disease (Craft et al., 2012) or the pituitary adenylate cyclase activating polypeptide to treat a variety of disorders including migraines and depression (Amin et al., 2014).

In order to fine-tune the methodology, a set of exploratory studies were performed in preparation for the experimental strategy. First, Wt and *Mct8*KO animals were treated with a highly concentrated solution of the prohormone T4 through intranasal delivery for a long-term period. This treatment had functional effects in the brain decreasing D2 activity in the cerebral cortex and increasing the expression of the T3-dependent gene *Aldh1a1* in the striatum of both Wt and *Mct8*KO animals. However, this treatment greatly increased T4 and in consequence T3 plasma levels in both Wt and *Mct8*KO animals in comparison to their basal controls. T4 plasma levels also increased after intranasal administration of T4 for a short-term period. From these exploratory studies it can be concluded that intranasal delivery of TH enters the blood torrent and therefore failed to identify if the TH mediated effects observed in the brain after T4 treatment were due to T4 reaching the brain directly after intranasal delivery or to the T4 reaching the brain from the systemic circulation as *Mct8*KO mice present the T4 transporter Oatp1c1 at the BBB. Additionally, in Mct8-deficiency, where the plasma levels of T3 are elevated, an increase in the plasma TH levels could be detrimental. For these reasons the focus was set on preventing TH from reaching the blood after intranasal delivery.

BSA has been suggested to work as a carrier for delivering drugs intranasally as it distributes throughout the whole brain and it does not reach the systemic circulation (Falcone et al., 2014). Taking advantage of the fact that BSA binds T3 with high affinity, the strategy was to administer T3 bound to BSA aiming to reach the brain without entering the blood. First, the

appropriate concentration of BSA that is able to bind the highest concentration of T3 suitable for intranasal delivery was estimated by a carefully designed radioactive BSA-T3 binding assay. Then, Wt mice were treated either with T3 alone, bound to BSA or T3 bound to BSA in the presence of the vasoconstrictor PHE. Neither the biding of T3 to the BSA nor the use of the vasoconstrictor were able to prevent or even reduce the T3 reaching the systemic circulation that increased around 10 times the basal T3 plasma levels.

Despite these initial discouraging findings, intranasal delivery offers so many advantages such as the potential to reach the brain bypassing the BBB in a non-invasive manner that it is deserving of further investigation to treat MCT8-deficiency neurological symptoms. The most immediate goal is to resolve whether TH administered intranasally is able to reach the brain directly. To achieve this T3 or T4 should be administered in animals deficient for their specific transporters, in other words, either *Mct8*KO mice should be treated with T3 or Oatp1c1 deficient mice should be treated with T4. In the absence of TH transporters, TH reaching the blood torrent might be prevented and even if it does reach the systemic circulations, the potential TH-mediated effects in the brain should be solely related to TH reaching the brain directly. To rule out the possibility that even in the absence of the specific transporter the administered TH is able to cross the BBB, a dosage of TH that produces an increase in the TH plasma levels comparable to that resulting from intranasal administration should be injected intraperitoneally and run in parallel as a control.

The outcome of this study highlights the difficulties of developing an effective treatment for MCT8 deficient patients. The coexistence of TH deficiency in the brain and TH excess in peripheral tissues means that the treatment should be effective in normalising the thyroidal status in the brain without worsening the peripheral hyperthyroidism.

III: Implications of the study and future directions

Considering the severe defects that MCT8 deficient patients present even before birth (López-Espíndola et al., 2014), an early diagnosis is essential to prevent unnecessary testing, to offer the affected families genetic counselling and most importantly to start with prospective treatments as soon as possible.

The study of TH economy during mouse foetal development will allow the understanding of physiological events regarding TH availability and action in brain and, in extent, will provide information about TH related pathological conditions. This will permit the development of

appropriate treatments and stablish timings to prevent, restore or at least palliate possible impairments. As studies with human samples are limited, it is necessary to research in animal models to understand TH action during brain development, as accomplished in the present work.

The possibility to perform *in utero* prenatal diagnosis of MCT8 (Ramos et al., 2011) offers the opportunity to start treatments during pregnancy by treating the mothers or by intra-uterine administration. Conceivably, TH analogues like DITPA or TRIAC, that have not been successful for improving the neurological defects of MCT8 deficient patients in postnatal stages, may be effective when administered in early stages of development. For instance, DITPA has been shown, in rodents, to cross the placental barrier and to normalise TSH levels and exert thyromimetic actions on the expression of T3-dependent genes in the pups' brain (Ferrara et al., 2014), although its use still has not been approved for pregnant women. Transplacental passage of TRIAC has been documented in pregnant women, as a possible therapy for foetal hypothyroidism, with no adverse reactions (Cortelazzi et al., 1999). However, the effects of TRIAC during Mct8 deficiency foetal development have not yet been assessed.

In humans, during the 13-20th week postmenstrual age of gestation there is D2 activity which leads to an accumulation of T3 in the cerebral cortex that follows an increase in the levels of T4 in the serum and in the T4 content of the cerebral cortex (Kester et al., 2004). Therefore, treating mothers carrying MCT8 deficient foetuses with LT4 or the TH analogue TETRAC, which can be metabolised into T3 and TRIAC respectively by D2 activity, might be appropriate. This thesis has proven that D2 activity is critical to provide T3 during mouse brain development, making the mouse a suitable experimental model to test these possibilities.

Finally, the findings regarding TRIAC treatment to *Mct8*KO mice can have a direct impact on the patients as TRIAC has been administered since 2014 to MCT8 deficient patients, some of them as part of a clinical trial that is testing TRIAC treatment in MCT8 deficiency (Iglesias et al., 2016, Groeneweg et al., 2016). So far the outcome of this treatment has revealed similar results to those obtained in *Mct8*KO mice in this thesis: a strong reduction in the circulating levels of T3 and T4 and also a slight improvement of peripheral markers. While lowering peripheral T3 levels is clearly beneficial for the patients, the extent of the consequences that might arise from low T4 serum levels is not known. Low T4 plasma levels might have a more detrimental effect in the brain of *Mct8*KO mice than in MCT8 deficient patients, as in the latter both T3 and T4 entry to the brain is believed to be impeded, nevertheless, the potential consequences of low T4 serum levels in humans should not be ignored.

CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. In the mouse, at least 50% of foetal T4 during late gestation is of maternal origin and maternal-foetal T3 transfer is tightly regulated, most probably at the placental level.

2. Regulation of transporter (*Mct8*, *Oatp1c1*, *Lat2*) or *Dio3* expression does not appear to play a role in maintaining foetal brain T3 homeostasis under a range of brain T4 values.

3. D2 activity is already present at E18 and is highly regulated at the post-translational level under changing T4 content in the brain. Conversion of T4 into T3 by D2 activity might be the only source of T3 during mouse brain development.

4. *Dio2* mRNA is expressed in the mouse brain at least from E15. Its expression in the meninges, the ependymal layer of the lateral ventricles and the choroid plexus suggests an important role of D2 at the BCSFB highlighting its relevance as a source of TH during early brain development. T4 transport across the choroid plexus along with D2 activity at the BCSFB might be key events in TH action in brain during embryonic development.

5. Despite the significant maternal T4 contribution to the total foetal T4 pool, under low levels of maternal T4 the foetus is able to trigger compensatory mechanisms in the brain to ensure an appropriate content of T3.

6. D2 activity is required to generate the TH excess in the brain of *Mct8*KO animals during perinatal stages of development and this hyperthyroidism is not due to increased TH transport directly from the systemic circulation.

7. Therapeutic doses of TRIAC in *Mct8*KO mice restore plasma T3 levels but severely decrease T4 levels which lead to a state of brain hypothyroidism with reduced T3 content. An increase in the plasma TRIAC levels after treatment is not sufficient to increase TRIAC levels in the brain and to promote the expression of T3-dependent genes in brain cells.

8. MCT8 delivery by AAV9 to the choroid plexus by IV but not ICV injection is crucial for its proper function by increasing T3 content. MCT8 mediates the entrance of T3 in the brain. The correct delivery route along with the appropriate AAV vector that restores MCT8 at the brain barriers are critical processes for an effective gene therapy to provide functional MCT8 in the brain of MCT8 deficient patients.

9. Administration of TH through intranasal delivery is probably not a treatment option because it results in increased TH levels in the systemic circulation even when it is administered bound to BSA and in the presence of a vasoconstrictor.

CONCLUSIONES

1. En el ratón, al menos un 50% de la T4 fetal durante las últimas etapas de la gestación es de origen materno y la transferencia materno-fetal de T3 está estrictamente regulada, probablemente a nivel placentario.

2. La regulación de la expresión de transportadores (*Mct8*, *Oatp1c1*, *Lat2*) o de la desyodasa *Dio3* no parece desempeñar una función en el mantenimiento de la homeostasis de T3 en el cerebro fetal, en presencia de valores variables de T4.

3. La actividad de la D2 está ya presente a E18 y se regula altamente a nivel postraducccional en presencia de valores variables de T4 en el cerebro. La conversión de T4 a T3 mediante la actividad de la D2 podría ser la única fuente de T3 durante el desarrollo fetal del cerebro de ratón.

4. El mARN de *Dio2* está presente en el cerebro de ratón por lo menos desde E15. La expresión de *Dio2* mARN en las meninges, la capa ependimaria de los ventrículos laterales y el plexo coroideo sugieren una importante función de la D2 en la barrera sangre-líquido cefalorraquídeo lo que destaca su relevancia como fuente de HT durante el desarrollo temprano del cerebro. El transporte de T4 a través del plexo coroideo junto con la actividad de D2 en la barrera sangre-líquido cefalorraquídeo podrían ser eventos claves en la acción de la HT durante el desarrollo fetal del cerebro.

5. A pesar de la considerable contribución de la T4 materna al conjunto total de T4 fetal, en condiciones de bajos niveles maternos de T4 el feto es capaz de activar mecanismos compensatorios en el cerebro para asegurar un contenido adecuado de T3.

6. La actividad de D2 es necesaria para generar el exceso de HT en el cerebro de ratones deficientes de Mct8 durante etapas perinatales del desarrollo y este hipertiroidismo no se debe a un aumento del transporte directo de HT desde la circulación sistémica.

7. Dosis terapéuticas de TRIAC administradas a ratones deficientes de Mct8 restablecen los niveles plasmáticos de T3 pero reducen drásticamente los niveles de T4 lo que genera un estado de hipotiroidismo cerebral con bajo contenido de T3. El aumento en

los niveles plasmáticos de TRIAC tras el tratamiento no es suficiente para aumentar los niveles de TRIAC en cerebro e inducir la expresión de genes dependientes de T3 en células cerebrales.

8. La localización de MCT8 en el plexo coroideo tras la inyección de AAV9 por vía intravenosa pero no por vía intracerebroventricular en cerebros deficientes del transportador facilita la entrada de T3 al cerebro. Una correcta vía de administración junto con un vector AAV adecuado son procesos críticos para restablecer MCT8 en las barreras cerebrales y para logar una terapia génica efectiva que proporcionen un MCT8 funcional en el cerebro de pacientes deficientes de MCT8.

9. La administración de HT mediante vía intranasal probablemente no sea una buena opción para el tratamiento de la deficiencia de MCT8 ya que genera un aumento en los niveles circulantes de HT incluso cuando es administrada unida a BSA y en presencia de un vasoconstrictor.

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degradation which regulates protein expression and oligomerization capacity. *Mol Cell Endocrinol,* 434**,** 278-87.

APPENDIX

Related publications

- Effect of Triiodothyroacetic Acid Treatment in Mct8 Deficiency: A Word of Caution.
  Bárez-López Soledad, Obregon Maria Jesus, Martínez-de-Mena Raquel, Bernal Juan, Guadaño-Ferraz Ana, and Morte Beatriz.
  Thyroid. 2016 May;26(5):618-26. doi: 10.1089/thy.2015.0388. Epub 2016 Feb 3.

- Adeno associate virus 9-based gene therapy delivers a functional monocarboxylate transporter 8 (MCT8) which improves thyroid hormone availability to brain of Mct8 deficient mice.
  Hideyuki Iwayama, Xiao-Hui Liao, Lyndsey Braun, **Soledad Bárez-López**, Brian Kaspar, Roy E Weiss, Alexandra M Dumitrescu, Ana Guadaño-Ferraz and Samuel Refetoff.
  Thyroid. 2016 Sep;26(9):1311-9. doi: 10.1089/thy.2016.0060. Epub 2016 Aug 23.

- Thyroid hormone homeostasis in the perinatal mouse brain.
  Soledad Bárez-López, Maria Jesus Obregon, Juan Bernal and Ana Guadaño-Ferraz.
  Manuscript in preparation for Cerebral Cortex.